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Relative mRNA Abundance in Bovine Tissues Related to Energy Metabolism.

BY ©

Gordon Kenneth Murdoch

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fulfillment of the requirements for the degree of :

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ABSTRACT

The physiological function and activity of all somatic tissue is a product of the specific genes that it expresses, as well as the pattern by which it alters its specific gene expression profile in response to the environment. We hypothesized that variability in parameters related to energy metabolism in ruminants is related to variability within tissue specific, gene expression profiles. Candidate genes were examined using RT-PCR to evaluate the relative mRNA abundance of these genes in several "metabolically active" tissues. The mRNA abundance was determined in *Biceps femoris*, cardiac muscle, liver, rumen papillae, abomasum, duodenum, mesenteric adipose, peri-renal adipose, and subcutaneous adipose tissue. The mRNA abundance was evaluated in the context of breed differences as well as acclimation to varied feeding level, diet type, ambient temperature, and also response to acute cold and acute feed restriction. The relative mRNA abundance was compared in individual animals with indices related to energy metabolism including, heat production, measured using indirect calorimetry, average daily gain, metabolizable energy intake, efficiency of energy retention and final carcass grade, where reasonable and applicable. As the first study involving a broad-spectrum evaluation of these candidate genes in the context of ruminant adaptive physiology, substantial variability in mRNA abundance was observed among animals and numerous relationships were described between tissue-specific mRNA abundance and the indices of energy metabolism. A link was observed between subcutaneous and skeletal muscle uncoupling protein mRNA abundance and chronic and acute cold adaptation. This project establishes the foundation for

future research regarding complex ruminant physiological adaptation strategies, related to tissue-specific alterations in gene expression in the context of production pertinent stressors.

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TABLE OF CONTENTS

CHAPTER 1 General Introduction

1.1 Introduction........1

1.2 Hypotheses........5

1.2.1 Hypothesis 1........5

1.2.2 Hypothesis 2........6

1.2.3 Hypothesis 3........6

1.2.4 Hypothesis 4........6

1.2.5 Hypothesis 5........6

1.2.6 Hypothesis 6........6

1.3 References Cited........7

CHAPTER 2 Review of Literature

2.1 Candidate Genes........8

2.1.1 Uncoupling protein-1........8

2.1.1.1 Control of Brown Fat Thermogenesis........11

2.1.1.2 Catecholamine Mediation of Brown Adipose Tissue........15

2.1.1.3 Characteristics of Thermogenin (UCP-1)........17

2.1.1.4 UCP-1 Activity........20

2.1.1.5 Regulation of UCP-1 Gene Expression........26

2.1.2 Uncoupling Protein-2........32

2.1.2.1 Characteristics of UCP-2........33

2.1.2.2 UCP-2 Tissue Expression, Activity and Regulation........33

2.1.3 Uncoupling Protein-3........44

2.1.3.1 Characteristics of UCP-3........45

2.1.3.2 UCP-3 Activity and Regulation........47

2.1.4 Leptin........54

2.1.4.1 Characteristics of Leptin........56

2.1.4.2 Leptin Activity and Regulation........57

2.1.5 Leptin Receptor........65
2.1.5.1 Leptin Receptor Characteristics........66
2.1.5.2 Leptin Receptor Activity........68
2.1.6 Neuropeptide Y (NPY)........73
2.1.6.1 Characteristics of NPY........73
2.1.6.2 NPY Activity and Regulation........76
2.1.7 Neuropeptide Y Receptor(s)........80
2.1.7.1 Characteristic of NPY Receptors........80
2.2 Summary of Review........83
2.3 References........85

CHAPTER 3 Uncoupling Proteins 2 and 3 mRNA Expression in Bovine Tissues and Their Relationship to Breed, Feeding Level, and Measurements of Energy Efficiency.

3.1 Introduction........108
3.2 Material and Methods........111
3.2.1 Animals and Treatments........111
3.2.2 Assessment of Energy Utilization........113
3.2.3 Gene Expression Studies........113
3.2.3.1 RNA Extraction........113
3.2.3.2 Reverse Transcription and Polymerase Chain Reaction....114
3.2.3.3 Statistical Analyses........116
3.3 Results........117
3.3.1 General Metabolic Responses........117
3.3.1.1 Digestibility........117
3.3.2 Breed Comparisons........118
3.3.3 Metabolic Response to Feeding........118
3.3.4 Variation in Heat Production Among Individual Animals Within a Breed........119
3.3.5 Gene Expression........120
3.3.5.1 Gene Expression Study of UCP-1, UCP-2, and UCP-3....120

3.4 **Discussion**..122
3.5 **References Cited**...135

CHAPTER 4 The mRNA Expression of Leptin, Neuropeptide Y and Receptors in Bovine Tissues and Their Relationship to Breed, Feeding Level and Measurements of Energetic Efficiency.

4.1 **Introduction**...147
4.2 **Material and Methods**...150
  4.2.1 Animal and Treatments..150
  4.2.2 Assessment of Energy Utilization....................................152
  4.2.3 Gene Expression Studies...153
    4.2.3.1 RNA Extraction..153
    4.2.3.2 Reverse Transcription and Polymerase Chain Reaction....153
    4.2.3.3 Statistical Analyses...156
4.3 **Results**..156
  4.3.1 Metabolic Data..156
    4.3.1.1 Digestibility...156
  4.3.2 Breed Comparisons...157
  4.3.3 Metabolic Response to Feeding...158
  4.3.4 Variation in Heat Production Among Individual Animals Within a Breed...158
  4.3.5 Gene mRNA Expression Data...159
  4.3.6 Breed and Feeding Level..160
  4.3.7 Comparison of Tissue Specific mRNA Expression and Metabolic Data...165
4.4 **Discussion**..166
4.5 **Reference Cited**..180

CHAPTER 5 The Effect of Diet Type, Level and Environment on UCP-2, UCP-3, Leptin Receptor and Neuropeptide Y Receptor mRNA abundance in Bovine Skeletal Muscle Biopsies

5.1 Introduction..196
5.2 Materials and Methods..197
  5.2.1 Animals and Management..197
    5.2.1.1 Animal Health and Training...198
    5.2.1.2 Animal Housing..198
    5.2.1.3 Diets..199
    5.2.1.4 Energy Balance and Metabolism Measurements............200
  5.2.2 Sub-component to Experiment...201
    5.2.2.1 Diet and Intake Effects..201
    5.2.2.2 Environment and Diet Effects.......................................201
  5.2.3 Statistical Analyses...201
5.3 Results..202
  5.3.1 Energy Metabolism Parameters...202
  5.3.2 Environment and Heat Production....................................204
  5.3.3 Results of Genetic Analyses...204
    5.3.3.1 Gene Expression Profiles in Muscle Biopsies...............204
5.4 Discussion..204
5.5 References Cited..211

CHAPTER 6 The Effects of Acute Cold Exposure and Acute Feeding Restriction on mRNA Expression In Bovine Tissues.

6.1 Introduction..227
6.2 Materials and Methods..231
  6.2.1 Animals and Management..231
    6.2.1.1 Effects of Acute Cold Exposure or Feed Restriction on Gene Expression in Steers..232
  6.2.2 Gene Expression Studies..232
    6.2.2.1 RNA Extraction..232
    6.2.2.2 Reverse Transcription and Polymerase Chain Reaction.....233
  6.2.3 Statistical Analyses...234
6.3 Results..234

6.3.1 Gene Expression Profiles……………………………………234
6.3.1.1 Leptin, Leptin Receptor and Neuropeptide Receptor…….234
6.4 Discussion……………………………………………………237
6.5 References Cited……………………………………………245

CHAPTER 7 General Discussion and Conclusion.

7.1 General Discussion…………………………………………255
7.1.1 Uncoupling Protein-1 (UCP-1)…………………………………255
7.1.2 Uncoupling Protein-2 (UCP-2)…………………………………256
7.1.3 Uncoupling Protein-3 (UCP-3)…………………………………258
7.1.4 Leptin……………………………………………………………261
7.1.5 Leptin Receptor…………………………………………………264
7.1.6 Neuropeptide Y (NPY)…………………………………………266
7.1.7 Neuopeptide Y Receptor………………………………………266
7.2 Experimental Limitations……………………………………268
7.3 General Conclusion…………………………………………270
7.4 References Cited……………………………………………271

Appendices General

Appendix One……………………………………………………273
Appendix Two……………………………………………………275
Appendix Three…………………………………………………279
Appendix Four……………………………………………………282

LIST OF TABLES

Table 3-1 Digestibility^{z} of DM, ME and protein.....................................142

Table 3-2 Breed.....................................143

Table 3-3 UCP2 Breed and Treatment.....................................144

Table.3-4 UCP2 and UCP-3 correlations with measures of energetic efficiency and heat production.....................................145

Table 3-5 Effect of breed and feeding level on mRNA expression of UCP2 and UCP-3 in tissues.....................................146

Table 4-1 Effect of breed and feeding level on mRNA expression of leptin, leptin receptor and NPY receptor in muscle.....................................188

Table 4-2 Effect of breed and feeding level on mRNA expression of leptin and NPY receptors in liver and GI tissues.....................................189

Table 4-3 Effect of breed and feeding level on mRNA expression of leptin and NPY receptors in adipose depots.....................................190

Table 4-4 Correlations with measures of energetic efficiency and heat production.....................................191

Table 4-5 Correlations with measures of energetic efficiency and heat production.....................................192

Table 4-6 Correlations with measures of energetic efficiency and heat production.....................................193

Table 5-1 Effect of diet and feeding level on parameters of energy intake, excretion and energy balance in steers.....................................217

Table 5-2 Effect of environment on energy intake and balance in steers.....................................218

Table 5-3 Effect of environment on heat production in steers during periods one (January) and one (February) in same year, showing progressive changes over time in the different environments......219

Table 5-4 Composition of diets fed during experiment 220

Table 5-5 Correlation's between gene expression analyses in muscle biopsies and indices of efficiency and heat production.....................................221

Table 5-6 Treatment Mean Leptin receptor mRNA abundance in skeletal muscle biopsies; interaction between period 1, 2, 3 & 4, environment, and diet……………………………………………222

Table 5-7 Muscle biopsy gene expression averages for inside, outside, roughage and concentrate treatments……………………………223

Table 6-1 Average and standard deviations of gene expression in acutely treated steers……………………………………………………251

Table 6-2 Average and standard error of gene expression in acutely treated steers………………………………………………………………252

Table 6-3 Averages and standard error of gene expression values in *post mortem* tissues from acute *antemortem* treated animals…………………………………………………………………253

LIST OF FIGURES

Figure 2-1 Sympathetic regulation of Brown adipose tissue......................14

Figure 2-2 Uncoupling protein-1 structure..18

Figure 2-3 Role of mitochondria in metabolism..................................24

Figure 2-4 Proposed models of UCP-1 proton transport........................25

Figure 2-5 Sympathetic up-regulation of UCP-1..................................27

Figure 2-6 Representation of UCP-1 gene activator element....................28

Figure 2-7 Human UCP-2 gene structural organization..........................34

Figure 2-8 UCP-2 up-stream promoter regions.....................................38

Figure 2-9 Diagram of mitochondrial establishment of proton electro-chemical gradient, and UCP uncoupling...................43

Figure 2-10 Human and mouse intergenic region of UCP-3 and UCP-2........46

Figure 2-11 Murine UCP-3 and UCP-2 exon boundaries..........................46

Figure 2-12 Schematic representation of UCP-3 gene promoter regulation by MyoD and retinoic acid...52

Figure 2-13 Exon organization of human Ob gene.................................56

Figure 2-14 Genomic structure of human Ob gene.................................57

Figure 2-15 Factors influencing leptin production in WAT.......................59

Figure 2-16 Leptin-NPY feedback under conditions of food restriction and food abundance..60

Figure 2-17 Diagram of Hypothalamic signaling under starvation and satiety conditions...62

Figure 2-18 Model of negative feedback regulation of adiposity.................66

Figure 2-19 Comparison of the leptin receptor isoforms..........................67

Figure 2-20 Model of signaling by OB-Rb...69

Figure 2-21 Schematic of human NPY gene, mRNA and protein precursor.....75

Figure 2-22 Model of Hypothalamic NPY pathway in control of energy balance...77

Figure 2-23 Model of integration of two long term adiposity signals in the regulation of systemic adiposity..79

Figure 2-24 Schematic of the human Y1 and Y5 gene cluster showing exons and alternative splicing……………………………………………..82

Figure 3-1 Image of Cardiac muscle UCP-2 amplicon (263 b.p. bottom) with G3PDH internal control (480b.p. top) separation on 2% agarose gel………………………………………………………140

Figure 3-2 Image of Cardiac muscle UCP-3 amplicon (499b.p. top) with G3PDH internal control (480b.p. bottom) separation on 2% agarose gel……………………………………………………...…141

Figure 4-1 Ethidium bromide stained image of separation of Mesenteric adipose leptin mRNA specific amplicon on 2% Agarose Gel……185

Figure 4-2 Ethidium bromide stained image of separation of leptin receptor mRNA specific amplicon on 2% Agarose Gel…………186

Figure 4-3 Ethidium bromide stained image of separation of NPY receptor mRNA specific amplicon on 2% Agarose Gel…………….……187

Figure 4-4 Histogram of quantifications of individual animals expression of leptin receptor mRNA in *biceps femoris* tissue………………..194

Figure 4-5 Histogram of quantifications of individual animals expression of NPY receptor mRNA in biceps femoris tissue………………...195

Figure 5-1 UCP3 (top 499b.p) amplicon in *biceps femoris* biopsy with G3PDH (bottom 480 b.p) amplicon as internal controlrun on a 2% Agarose Gel……………………………..……………………...213

Figure5-2 UCP2 (bottom 263bp) amplicon in *biceps femoris* biopsy with G3PDH (top 480bp) as internal control run on a 2% Agarose Gel…………………………………………………….....…214

Figure 5-3 Leptin receptor amplicon (bottom 379 bp) in *biceps femoris* biopsy with G3PDH internal control (top 480 bp) run on a 2% Agarose Gel…………………………………………………………...215

Figure 5-4 NPY receptor amplicon (bottom 313bp) in *biceps femoris* biopsy with G3PDH internal control (top 480bp) run on a 2% Agarose Gel…………………………………………………………...216

Figure 5-5 Relationship between daily energy retention (ER) and metabolizable intake (MEI) in steers housed in two environments during winter……………..…………………….224
Figure 5-6 Comparison of mean heat production values for inside and outside housed animals over two consecutive periods…………...225
Figure 5-7 Comparison of heat production values over periods 1 &2 for two planes of intake……………………………………..…..226
Figure 6-1 NPY receptor amplicon bottom in subcutaneous adipose tissue with G3PDH top, run on a 2% Agarose Gel……………………...248
Figure 6-2 Leptin receptor amplicon bottom in subcutaneous adipose tissue with G3PDH top, run on a 2% Agarose Gel……………….249
Figure 6-3 Leptin amplicon (460bp) in subcutaneous adipose tissue, run on a 2% Agarose Gel……………………………………….250
Figure 6-4 Comparison of mean leptin mRNA expression in subcutaneous adipose tissue between carcass grades……………...…………254

List of Abbreviations

ACTH	adrenocorticotropin hormone
ADF	acid detergent fiber
ADG	Average daily gain
ADP	Adenosine Diphosphate
AIA	acid insoluble ash
ATP	Adenosine triphosphate
BAT	brown adipose tissue
BF	biceps femoris skeletal muscle
BW	body weight
C	celsius
cDNA	complimentary deoxyribonucleic acid
CP	crude protein
DE	digestible energy
DEI	digestible energy intake
DM	dry matter
DMEI	delta mei
DMER	delta MER
DMER/DMEI	efficiency of energy retention
DMI	dry matter intake
DNA	deoxyribonucleic acid
ER	energy retention
EXP	Experiment
FHP	fasting heat production
GE	gross energy
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
H	hour(s)
HP	heat production
KG	kilograms
LEP	leptin

LEP-R	leptin receptor
M	Maintenance
MA	mesenteric adipose tissue
MBW	metabolic body weight
ME	metabolizable energy
ME_M	Metabolizable energy of Maintenance
MEI	Metabolizable energy intake
MJ	mega joules
mRNA	messenger ribonucleic acid
NDF	neutral detergent fiber
NO	nitric oxide
NPY	Neuropeptide Y
NPY-R	Neuropeptide Y receptor
PCR	polymerase chain reaction
PKA	protein kinase A
PVMT	peripheral vasomotor tone
RNA	ribonucleic acid
ROS	reactive oxygen species
RPM	revolutions per minute
RT-MMLV	reverse transcriptase-moloney murine leukemia virus
RT-PCR	reverse transcription-polymerase chain reaction
SA	subcutaneous adipose tissue
SD	standard deviation
SE	standard error of the mean
TAQ	thermous aquaticus DNA polymerase
TIMP	tissue inhibitor of metalloprotease
UCP-1	uncoupling protein-1/thermogenin
UCP-2	uncoupling protein –2
UCP-3	uncoupling protein –3
WAT	white adipose tissue

CHAPTER 1

General Introduction

1.1 Introduction

It is well understood that the efficiency of conversion of feed by livestock can influence the overall profitability of the livestock production system. Given the variability in efficiencies of energy utilization in animals, housed in similar environments, fed similar planes of nutrition and composition of diets, it is apparent that the energetic efficiency may be related to genetic traits and, more specifically, to the expression of these traits in metabolically active tissue. In July 2001, the Alberta Beef cattle population was 5.82 million head and the total production in terms of slaughtered and processed beef in 2001 was 2.33 million animals. This represented about 62.46% of the total Canadian production (Canadian Cattleman's Association website, www.cattle.ca./canfax statistics pdf, 2002). These numbers emphasize that development of a system for determining and selecting efficient beef cattle, within Alberta alone, could represent substantial economic significance for producers even if a net savings of as little as $5.00/ head were achieved. Though, in the past, typical breeding strategies have relied on selection protocols incorporating only phenotypic animal traits there is a trend to include more genotypic as well as physiological traits into herd breeding stock selection. The potential for establishing a genetic marker that correlates well with an animal's potential for efficient energy utilization without sacrificing meat quality parameters nor being detrimental to important adaptational qualities, is appealing to livestock producers. Marker-assisted selection could thus be used to optimize production efficiency, as well as determine animals that could be earmarked for breeding stock. The success of such a selection program would rely on the ability for the marker to be measured early and yet reliably predict an animal's relative efficiency throughout the typical animal production span. In order to determine which gene(s) may serve as such reliable and accurate determinants of livestock efficiency, a better understanding of candidate gene physiology in cattle is required.

With the objective of understanding the relationships between tissue specific gene expression and indices of feed conversion efficiency in mind, several candidate genes were chosen for evaluation in various metabolically active beef cattle tissues. The expression profiles for these genes were determined and compared to individual animal heat production as well as dietary intakes and feed conversion efficiencies. Further, it was decided to evaluate these genes in the context of different breeds (Charolais, Angus, Brahman/Angus cross and Kinsella mixed beef composite), different feeding levels (1.2M, 2.2M and ad libitum), different dietary composition (90% w/w hay roughage, 90% w/w barley concentrate) and different ambient temperatures (cold acclimated, thermoneutral and acute cold); so as to most effectively represent some of the typical Alberta production conditions. Taking into consideration existing scientific understanding of several gene products in other species as well as their known or hypothesized relevance to metabolism and dietary intake, certain genes were determined to be good candidates for potentially influencing relevant physiological pathways related to production efficiency.

Given that energy can neither be created nor destroyed, but rather only transformed, it is well accepted that production relies on the conversion of nutrients (Intake energy) into proteins, carbohydrates, and lipids that serve as building blocks for the growing animal. While abiding the underlying laws of thermodynamics we do observe variations in the efficiencies of nutrient conversion by livestock, into anabolic growth. Furthermore it is well understood that certain physiological processes demonstrate net efficiencies of zero, that is no real work is performed though chemical energy is transformed into heat. An example of such a process is, shivering thermogenesis, where the futile contraction and relaxation of skeletal muscles utilizes ATP stores and dissipates this chemical potential energy as thermal energy, or heat.

Just as in humans where certain individuals are predisposed to eat more than others and this intake may not always be exactly correlated with body composition, so it is observed in beef production. Some animals ingest more than other animals of similar age, size and breed and, as stated earlier, there is variability in the conversion of nutrient intake into body reserves for growth, lean tissue deposition or fat deposition. The regulation of an animal's satiety, or conversely its impulse to ingest nutrients may both be important variables in the regulation of that animal's metabolic status. The discovery of leptin, its physiologic regulation of satiety and its relationship to obesity in animals with either leptin deficiency, or leptin insensitivity, suggest it as a candidate for involvement in mediating energy metabolism in animals (Hossner, 1998). Likewise, the positive influence of hypothalamic neuropeptide Y on food intake and the expression of its receptors in numerous tissues suggest that it could also be a candidate for mediation of energy metabolism.

Discovery of uncoupling protein-1 (thermogenin), its role in brown fat adaptive thermogenesis as well as the identification of related proteins (UCP-2 and UCP-3) in several other tissues made these candidates for influencing livestock metabolism and efficiencies. If the expression of these genes influences the extent to which the proton motive force is coupled to ATP synthesis they may well affect an animal's inherent heat production and therein its energetic efficiency. Hence, variations among an individual animal's tissue specific expression of uncoupling protein genes may be associated with slight differences observed in heat production values for different individual animals. Furthermore, adaptation of transcription of these genes may correlate with elevated thermogenic capacity under influence of cold treatment or variations in above maintenance planes of nutrition. Our candidate genes were thus established as; leptin, neuropeptide Y, leptin receptor, neuropeptide Y receptor, uncoupling protein-1, uncoupling protein-2, and uncoupling protein-3. These candidate genes will be annotated in more detail, in forthcoming sections of this literature review.

Several tissue types were chosen as important depots for analyses relevant to these candidate genes' expression profiles. One obvious choice was skeletal muscle since it is a metabolically active tissue that can represent as much as 35% of an animal's body weight. Further, skeletal muscle is the marketable commodity related to beef production making it essential to our study. We selected the *biceps femoris* muscle, as a representative muscle due to its accessibility and since it is known to be of mixed-fiber type. Three adipose depots; subcutaneous, mesenteric and perirenal, were sampled due to their potential relationship to an animal's nutrient sensing pathways and metabolic activity. The rumen papillae, abomasum, duodenum and liver were selected for their individual involvement in nutrient processing, their high nutrient exposure as well as their respective high metabolic activities. Finally, cardiac muscle was also sampled due to its adaptability and continuous contribution to both an animal's basal metabolic rate and adaptive physiology. The expression of these candidate genes in these respective tissues has been determined to some extent by previous research, though most of this research involved animal species other than cattle. Our study would inherently generate bovine-specific information that would capitalize on physiological and genetic information gained by other research while determining gene expression profiles in our selected bovine tissues, changes in expression induced by treatments as well as breed and individual animal variability.

1.2 Hypotheses

The hypotheses that we endeavoured to test in this research project include the following:

1.2.1 The mRNA abundance will vary in accordance with the specific tissue examined, between individual animals, and between different breeds of cattle that are phenotypically different.

1.2.2 Varied mRNA abundance in specific tissues relate to their physiological function, state, and relative adaptation to stress that challenges their ability to maintain a homeorrhetic state.

1.2.3 The tissue-specific mRNA abundance of our candidate genes will be affected by plane of nutrition and diet type.

1.2.4 The mRNA abundance will show tissue-specific adaptation and correlation to environmental temperature, heat production, and indices of metabolic efficiency in growing steers.

1.2.5 The mRNA abundance of our candidate genes in specific tissues will change due to acute or chronic cold exposure of our steers.

1.2.6 The mRNA abundance of our candidate genes in specific tissues will change due to acute feed restriction.

1.3 References Cited

Canadian Cattleman's Association website, www.cattle.ca./canfax statistics pdf, 2002

Hossner, K.L. (1998) Cellular, molecular and physiological aspects of leptin: Potential application in animal production. Can J. Anim. Sci. 463-472.

CHAPTER 2

Review of the Literature

2.1 Candidate Genes

2.1.1 Uncoupling protein-1

It is prudent to briefly review early studies that involved the examination and characterization of one particular tissue directly involved in the eventual discovery of thermogenin (uncoupling protein-1): brown adipose. In a recent review by Rial et. al. (2001) he attributes the first citation and recognition of a distinct tissue present in the interscapular region of the marmot to Conrad Gesner in 1551. The tissue was believed to be an endocrine gland and further an extension of the thymus. It was referred to early as the "hibernating gland", based on its observed presence in hibernating species, but was initially thought to be a source of water during hibernation (Johansson, 1959). In the late 19th century it was histologically identified as a modified fatty tissue and subsequently referred to by such terms as "primitive fat" and "lipoid gland" (Rial et. al., 2001).

Accompanied by the early biological understanding of homeothermic animals it became apparent that the regulation and maintenance of a reasonably homeostatic body core temperature, in the face of varied ambient temperatures, requires both sensation and response to thermal conditions, hence the homeorrhetic philosophy of adaptive thermogenesis. Not only must a warm homeotherm have the capacity to dissipate heat, a contrastingly cold homeotherm must have the capacity to acquire heat or increase its heat production or thermogenesis. Although there was a biochemical understanding of the conversion of chemical energy contained in ingested nutrients into other forms of energy utilized by the animal including thermal energy, a grasp of physiological mechanisms that were available to increase thermogenesis was limited. Max Kleibers' release of "The Fire of Life- an introduction to animal energetics" provided an invaluable reference in terms of measuring heat production in animals and the value and reliability of such measures in representing the chemical "Katabolism" within animals (1961).

Still in the early nineteen-sixties two independent scientific reports established a functional role for brown adipose in thermogenesis when it was shown that brown fat was the thermogenic effector in arousing hibernators (Smith and Hock, 1963) and a thermic tissue in new-born rabbits in response to the cold (Dawkins and Hull, 1963). This physiological role for brown adipose became broadly accepted when a review in 1969, by Smith and Horwitz established a clear link between brown fat and non-shivering thermogenesis as they outlined their research regarding non-phosphorylating respiration in brown adipose mitochondria. These reports were corroborated by Lindberg et. al. (1967) regarding brown adipose mitochondrial energy transfer that was estimated using the P/O ratio in homogenized tissue. Although this ratio was typically about 1.8 in liver homogenates in brown fat homogenates it was usually near zero, representing nearly complete uncoupling of cellular respiration (oxygen uptake) from oxidative phosphorylation, in brown adipose homogenates. While examining the mitochondria from rat brown adipose an increase in passive ion permeability was observed in the presence of nucleotides (Nicholls and Lindberg, 1973), this supported an earlier association made between guanosine-triphosphate and coupling of oxidative phosphorylation (Rafael et. al., 1969). Rafeal also demonstrated that long-chain fatty acids appeared to increase brown fat mitochondria uncoupling of oxidative phosphorylation, and that quenching of fatty acids by addition of albumin increased the degree of brown fat mitochondrial coupling. These discoveries resulted in twenty years of research regarding the physiology behind this and other examples of whole animal adaptive thermogenesis. For instance, following cold exposure of newborn rats, an increase in purine nucleotide binding, accompanied by a delayed but subsequent increase in thermogenesis was observed in brown adipose tissue mitochondria (Desautels et. al., 1978) and further obese rats failed to increase nucleotide binding in response to cold (Himms-Hagen and Desautels, 1978). A year later a link between brown fat and diet-induced thermogenesis was reported (Rothwell and Stock, 1979). The aforementioned research had established that brown fat was involved in adaptive thermogenesis induced by either a reduction in ambient temperature or an increase in nutrient intake. Moreover, these adaptive effects paralleled observed changes in nucleotide binding. Additionally, and related to the concept of whole animal energy balance, deficits in brown adipose adaptive thermogenesis upon cold exposure were associated with at least one form of obesity in rodents.

Future research directed at understanding brown fat thermogenesis was subsequently facilitated by two important discoveries and publications. The first of these was the identification of a purine nucleotide binding protein in the inner membrane of brown fat mitochondria (Lin and Klingenberg, 1982), and the second was the extraction of this 32,000 MW uncoupling protein from these mitochondria (Rial et. al., 1983). Nicholls and Locke (1984), immediately included aspects of these discoveries in their review of thermogenic mechanisms in brown fat. A key aspect of this review, turned out to be the revelation that although an increase in total purine nucleotide binding was well correlated with increased adaptive thermogenesis in response to chronic cold exposure, the addition of these nucleotides inhibited the extent of the observed uncoupling or proton "short-circuit".

2.1.1.1 Control of Brown Fat Thermogenesis

In order for any adaptive thermogenic response mechanism to be practical in terms of increasing an animal's energy expenditure for the physiological purpose of generating more heat to keep an animal warm, the onset of such a mechanism should be inducible through cold-exposure of the animal. As mentioned earlier, the association between brown adipose heat production and thermogenesis in hibernating species was made in the late sixties. It was also known by this time that brown adipose tissue activity was under the influence of its vast sympathetic innervation, as well as lipid mobilization and oxidation of fatty acid substrates. In vitro experiments on isolated brown adipocytes by Prusiner et. al. (1968), demonstrated that respiration in these cells was stimulated by both catecholamines and fatty acids. Moreover these two effects were separate based on the observed inhibition of the catecholamine stimulation by propranolol (β-adrenergic antagonist) but no decrement in the fatty acid-induced cellular respiration. Given that the hypothalamic center receives afferent sensory input that relates the sensed or perceived ambient (cutaneous) temperature, and that the hypothalamic temperature center responds with sympathetic efferent activation in response to cold, it is clear that this is an important component of adaptive thermoregulation. Sympathetic activation, especially in brown fat fibres, results in release of catecholamine neurotransmitters at the effector organ. Catecholamines are well documented to be activators of catabolic reactions including lipolysis and glycolysis, via ligand-receptor interactions with β-adrenoceptors located on effector organ cell surface. Adrenoceptors are coupled to G-proteins and influence second messenger production, such as an increase in cAMP due to G-protein stimulation of adenylate cyclase. The first proposal for the effects of catecholamines in terms of increasing brown fat thermogenesis was a futile cycle of substrate breakdown and resynthesis. This theory persisted until more circumstantial evidence suggested that thermogenin was key to the brown fat heat production, and a simple catabolic-anabolic cycling was inadequate. Of obvious importance to the more modern theory was the association between increased UCP-1 expression and cold ambient temperatures, which will be reviewed later.

There are both acute and chronic responses of brown adipose to cold exposure that are relevant to this tissue's thermogenic capacity. Chronic adaptation of brown adipose, induced upon cold exposure, includes; increased recruitment of adipocytes in fat depots to differentiate into brown fat adipocytes (Cannon & Nedergaard, 1983, Bukowiecki et. al., 1986,), hyperplasia of existing brown adipose tissue (Bukowiecki et. al., 1983, Trayhurn et. al., 1987), increased mitochondrial biogenesis and various increases in specific gene transcripts. More acutely, observations of increased lipolysis are attributed to sympathetic release of noradrenaline, which acts on surface adrenoceptors to elevate cAMP thereby activating protein kinase A, which in turn activates hormone-sensitive lipase which breaks down triglycerides into free fatty acids. This relationship was established earlier by work on isolated Hamster brown fat cells illustrating direct relationships between norepinephrine-induced cAMP with both observed increases in respiratory rate and lipolysis (Petterson and Vallin, 1976).

Further acute increases in brown fat UCP-1 activity have been well established, and appear to involve both adrenergic stimulation as wells as fatty acid activation (elevated by the increase in lipolysis), in response to cold stimulated sympathetic activation. Additionally, though conversely, in cold-acclimated rats returned to thermoneutral environments (de-acclimation) RT-PCR mRNA analyses (Reichling et. al., 1987) and Western Blot protein analyses (Peachey et. al., 1988) demonstrated a loss of brown adipose tissue uncoupling protein-1. This supports the premise of a direct influence of ambient temperature on brown adipose UCP as manipulation of temperature results in predictable changes in the quantity of UCP-1. Thus the expression of brown adipose UCP-1 is bi-directionally influenced by ambient temperature at the transcriptional level. There is also evidence showing that post-transcriptional regulation of UCP-1 mRNA is affected by cold exposure. Using actinomycin D to inhibit transcription after either cold exposure (16 hrs or 48 hrs) or thermoneutral treatment in the presence of ^{32}P-dCTP, the decay of mature transcripts of UCP mRNA, were decreased, by both cold treatments. This mRNA stabilization was observed as an increase in mRNA half-life from an average of 16hrs in thermoneutral to 47 hrs in cold-treated rats (Rehnmark et. al., 1992). It is implied that such an increase in mRNA stability would in effect result in increased translation of uncoupling protein.

These findings yield a plausible model, representing the physiological process mediating increased non-shivering thermogenesis upon cold exposure, that keys on the hypothalamic coordination of afferent thermal sensation with the sympathetic activation of brown adipose. The brown adipose responds to catecholamine stimulation of its cell surface adrenoceptors to induce both acute and chronic changes in its metabolic status (Summarized in the following figure, borrowed from Lowell and Spiegelman, 2000).

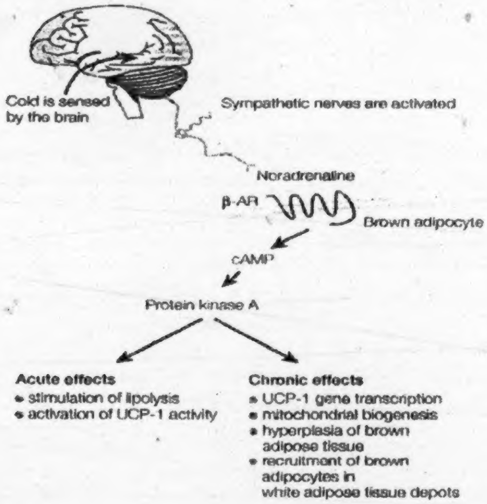
Cold is sensed by the brain
Sympathetic nerves are activated
Noradrenaline
β-AR
Brown adipocyte
cAMP
Protein kinase A
Acute effects
• stimulation of lipolysis
• activation of UCP-1 activity
Chronic effects
• UCP-1 gene transcription
• mitochondrial biogenesis
• hyperplasia of brown adipose tissue
• recruitment of brown adipocytes in white adipose tissue depots

Figure 2-1 Sympathetic regulation of Brown adipose tissue

2.1.1.2 Catecholamine Mediation of Brown Adipose Tissue

Armed with widespread evidence for the significant contribution of thermogenin in brown fat thermogenesis, research began to focus on specific regulation of brown fat UCP-1 expression. The availability of sympathomimetic and sympathospecific ligands allowed more detailed investigation of the signal transduction pathways involved in brown fat mitochondrial thermogenesis. Given that noradrenaline had been shown, in numerous studies, to be the endogenous sympathetic activator mediating brown fat thermogenesis and that these effects were a result of interaction with β-adrenergic receptors, Puigserver et. al. (1996) sought to determine which type of β receptor was responsible for this signaling mechanism. This research team treated BAT primary cultures with prenalterol (selective β-1 agonist) or salbutamol (selective β-2 agonist) or BRL 37344 (selective β-3 agonist) and found that all adrenergic ligands were able to induce increases in both UCP mRNA and protein expression (Puigserver et. al., 1996). Moreover this group established that the most potent of these adrenergic agonists, as established by IC_{50}, was the β-3 agonist (BRL37344) and that this ligand stimulation also induced the highest maximum UCP-1 protein expression. Although these findings directly contradict the finding by Bronnikov et. al. in 1992, where they publish that β-1 and not β-3 adrenoceptors were the most effective initiators of UCP-1 expression the models utilized in these two studies were different. It is quite possible that the use of non-confluent brown adipose primary cell cultures in Bronnikov's study was the cause of this disparity. The non-confluent cell cultures may not express the same receptor subtypes, and ratios of subtypes, as confluent cells. It is my perception that the confluent cultures used in Puigservers study are a more representative model of the in vivo tissue. By utilizing selective ligands for various adrenoceptors a better profile of the signaling cascade involved in regulating UCP expression has been obtained. The effects of catecholamine signaling on brown adipose have been studied in more detail than is illustrated in the previous figure and in fact not all of the chronic effects are regulated by PKA-coupled signaling pathways. Using fetal rat brown adipocytes, it was demonstrated that extracellular regulated kinases (ERKs) were activated by β-adrenergic, α-1 and α-2 adrenergic stimulation and this induced brown adipose cell growth, and that this effect was independent of the protein kinase A cascade (Valladeres et. al., 2000). The increase in brown adipose tissue observed is a combination of both hyperplasia and hypertrophy, thus increasing the number of cells and cell size. The increase in cell size allows the storage of more energy reserves, in the form of lipid content, which offers additional means through which to increase thermogenesis with the activation of lipase activity and generation of elevated free fatty acids in association with the increases in UCP-1 expression.

In summary, catecholamines influence the recruitment of new brown adipocytes, induce an increase in size of existing BAT cells and through increased UCP-1 expression, and increased lipolysis, elevate the thermogenic capacity of this tissue. The sympathetic innervation of this tissue, coupled with its inherent expression of sympathetic responsive cell surface receptors, allow BAT to be an effective source of non-shivering thermogenesis.

2.1.1.3 Characteristics of Thermogenin (UCP-1)

Thermogenin, or uncoupling protein 1, is a 32 kDa inner mitochondrial membrane protein, first identified using photo-affinity labeling in 1978, by Nicholls research team (Heaton et. al., 1978). This protein was subsequently purified from hamster (Lin and Klingenberg, 1980) and rat (Ricquier et. al., 1982) and the amino acid sequence was determined (Aquila et. al., 1985) and cloned as cDNA (Bouillaud et. al., 1985; Jacobsson et. al., 1985) three years later. It is a member of the mitochondrial carrier protein family, which includes citrate, ATP/ADP translocator and oxoglutarate/malate carriers. This family of transmembrane proteins have a tripartite structure in that there are three, internal, homologous domains roughly 100 amino acids long, domains within the protein sequence that are homologous regions. These are associated in the tertiary structure of the membrane protein such that they form a pore or channel. UCP-1 has six α-helical hydrophobic transmembrane regions, allowing its stable positioning within the inner mitochondrial membrane, with both its carboxy- and amino- terminal ends located in the mitochondrial intermembrane space. UCP-1 is believed to resemble other members of its carrier family and function as a homodimer. These structural dynamics are illustrated in the following figure taken from a review by Kozak and Harper (2000).

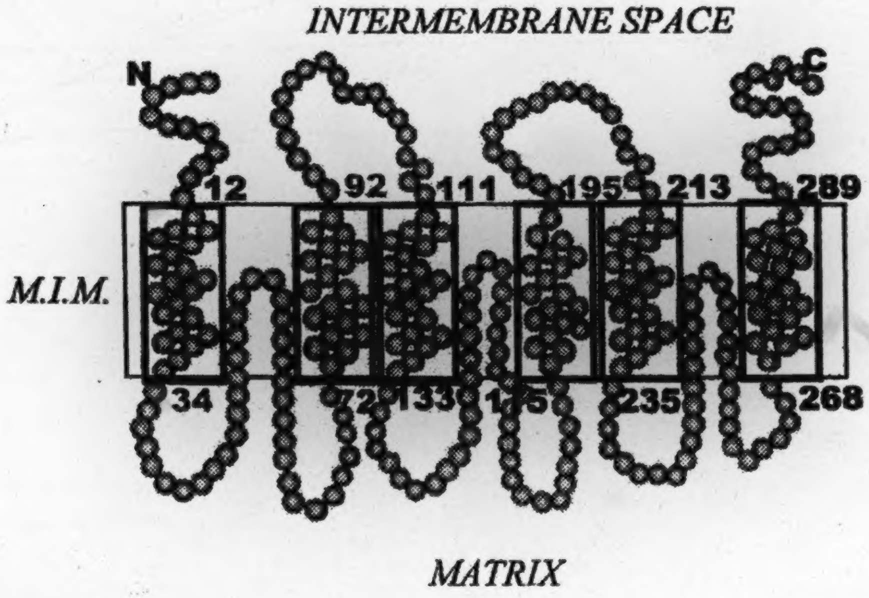

Figure 2-2 Uncoupling protein-1 structure

The UCP-1 gene is located on chromosome 17 in cattle (chromosome 4 in humans), and is comprised of six exons (1 per transmembrane domain) that encode for a 307 amino-acid sequence. There is no post-translational processing of the 307a.a. protein, but several amino terminal residues are involved in the mitochondrial inner membrane targeting and insertion.

The synthesis of ATP in mitochondria is driven by the electron transport chain and its inherent transfer of electrons through oxidation of reduced substrates (mainly NADH and FADH2) and concomitant generation of a proton gradient across the mitochondrial innermembrane. The abundant protons within the intermembrane space, generate a proton motive force that can be utilized since free energy is released while protons pass down their electrochemical gradient through ATP synthase into the mitochondrial matrix. The free energy released from this exergonic reaction can be captured in a high-energy bond between inorganic phosphorous and ADP, to yield ATP. It was recognized early that inhibition of ADP phosphorylation did not cessate mitochondrial oxygen consumption indicating that the coupling of this reaction was imperfect. The presence of proton leaks that can dissipate the proton motive force without ATP synthesis in mitochondria yield a release of free energy that is in the form of exergonic heat (Smith et. al., 1966). Uncoupling protein-1 can enhance the extent of "uncoupling", ergo it's name, and as such results in increased thermogenesis in these BAT mitochondria. The uncoupling process must be well regulated so as not to starve the cells of energy reserves which could result in cell death as is observed with other chemical uncouplers. The exact mechanism by which uncoupling protein dissipates the proton motive force is strongly debated and several conceptual models have been presented by the various factions of this debate. It has yet to be determined whether it is direct proton transport, anion transport (specifically Chloride anion, Jezek & Garlid, 1990) or fatty acid transport that is key to the functionality of UCP-1-mediated proton gradient dissipation, or whether it is a combination of several or all of these models. Furthermore, the regulation of this uncoupling remains somewhat debated, though most accept that it is influenced by the presence of fatty acids (activation) and nucleotides (inhibition). The following section will review some of the proposed mechanisms for UCP-1-mediated proton transport as well as some of the proposed domains for regulation of UCP-1 protein.

2.1.1.4 UCP-1 Activity

It was in the late sixties that nonphosphorylating respiration was observed and reported in rat mitochondria (Smith et. al., 1966), and this uncoupling was influenced by catecholamines (Prusiner et. al. 1968), long chain fatty acids and purine dinucleotides (Rafael et. al., 1969). With the discovery of the mitochondrial uncoupling protein as the site of this energy dissipation (Heaton et. al., 1978), and further that the relative amount of uncoupling protein expressed in brown fat mitochondria corresponds to the degree of observed dissipation of the proton gradient across the mitochondrial inner membrane; UCP-1/thermogenin became the focus of research related to non-shivering thermogenesis. Validation of the integral role of UCP in this physiological role has accumulated with the advent of more powerful molecular analyses. Perhaps the most direct was the use of an expression vector for UCP-1 to yield the functional expression of UCP-1 in mammalian Chinese hamster ovary (CHO) cells which resulted in mitochondrial alterations sufficient that the transfected cells' mitochondria were now functionally similar to those in brown fat (Casteilla et. al., 1990). The CHO cells remained viable since their intrinsic abundance of nucleotides served to attenuate the degree of UCP-1-mediated uncoupling allowing for sufficient cellular ATP synthesis. Additionally, the expression of UCP-1 in yeast mitochondria served to verify that this protein was responsible for disrupting the coupling of the proton motive force to ATP synthesis (Bathgate et. al., 1992). In fact early yeast clones, expressing UCP-1 died, unless a galactose-dependent promoter capable of mediating the amount of UCP expressed was included in the plasmid. It was demonstrated that the expressed UCP proteins were targeted and inserted into the inner mitochondrial membrane, in both cells, and functioned in the yeast by dissipating the proton gradient generated by the concomitant transport of protons into the inner membrane space during activity of the electron transport chain. With an appreciation that purine nucleotides (ATP, ADP, GTP, and GDP) had the capacity to reduce the uncoupling associated with UCP-1 and without such regulation excessive UCP-1 mediated uncoupling was lethal to cells. The establishment of a high expression system for UCP-1 in yeast cells allowed the evaluation of uncoupling protein physiology through studies employing site-directed mutagenesis.

Several research groups sought to determine the nature of this purine nucleotide inhibitory effect. Bouillaud strategized that since UCP-1 was a member of the mitochondrial membrane carrier family that includes ADP/ATP translocator (a specific carrier of these nucleotides) that perhaps domains of homology between these transporters may correspond to loci of nucleotide action. Bouillaud found that a region of high homology between these two carriers was conserved in the C-terminal domain of the protein (amino acids 261-269 in the rat) and proposed that the nucleotide interaction occurred in this region (Bouillaud et. al., 1986). Interestingly this C-terminal domain is also 80% conserved in UCP-2 and UCP-3 homologues, which will be discussed in subsequent sections of this literature review. The use of site-directed mutagenesis was employed to determine the domain of nucleotide binding to UCP-1, and it was discovered that deletion of amino acids 267-269 in rat UCP-1 resulted in a functional uncoupler that was not sensitive to nucleotide inhibition (Bouillaud et. al., 1994). The complete deletion of this domain resulted in a mutant protein that had a pore-like property, diminishing the selectivity previously observed in uncoupling protein. Therefore this C-terminal homology was at least in part related to the gating of the normal functional UCP. In addition three arginine residues (Arg^{83} Arg^{182} and Arg^{276}) were later found to be more specific key amino acids in purine nucleotide regulation of UCP-mediated uncoupling (Modriansky et. al., 1997). Moreover, it was observed that not all of these mutants lost the capacity to bind the nucleotides. The conversion of Arg^{83} to Gln and the mutation of Arg^{182} to Thr both disrupted binding and UCP inhibition but mutation of Arg^{276} to Leu allowed nucleotide binding but removed the capacity of this binding to inhibit UCP (Modriansky et. al., 1997). This group suggests that interaction of polyanionic nucleotides with the positively charged arginine residues sterically inhibits the uncoupling protein, and that Arg^{276} only assists inhibition of UCP activity, by resulting in a "tight conformation" that doesn't occur when the nucleotides bind to the $leucine^{276}$ mutant. It turns out that these three residues are conserved in UCP-2 and UCP-3 homologues, but are absent in a human short isoform of UCP-3, UCP-3s.

It was known that the inhibition by purine nucleotides is affected by pH. Specifically, that as the pH increases the binding of nucleotides, and inhibition by their binding, decreases (Klingenberg, 1988, and Huang and Klingenberg, 1995). This observed pH effect was found to be greater with nucleoside triphosphates than the diphosphates, and it was suggested that the protonation of an acidic residue (glutamate or aspartate) is a prerequisite for the nucleotide binding. Again using site-directed mutagenesis, the amino acid residues responsible for the pH mediation of the nucleotide inhibition of UCP were determined. It was found that Glu^{190} was the pH sensor for nucleotide binding (Winkler et. al., 1997) and that another residue, His^{214} was critical for only the pH-sensitive nucleoside triphosphate inhibition (Echtay et. al., 1997).

Through the use of site-directed mutagenesis again, it has been revealed that two key amino acid residues that are important to proton transport by UCP-1 are the histidine residues located in positions 145 and 147. These histidine residues are not conserved in other UCP homologues however it is speculated that D^{27} (Aspartate residue), which is conserved in all uncoupling proteins, is also important in proton transport.

Prior to delving into the mode of action of uncoupling protein it may be useful to consider a general perspective of the mitochondrial electron transport chain, some inner mitochondrial protein carriers, including UCP, and sources of reduced substrates important in the generation of the intermembrane proton motive force. The following figure illustrates these mitochondrial elements, it was borrowed from a review by Lowell and Spiegelman (2000).

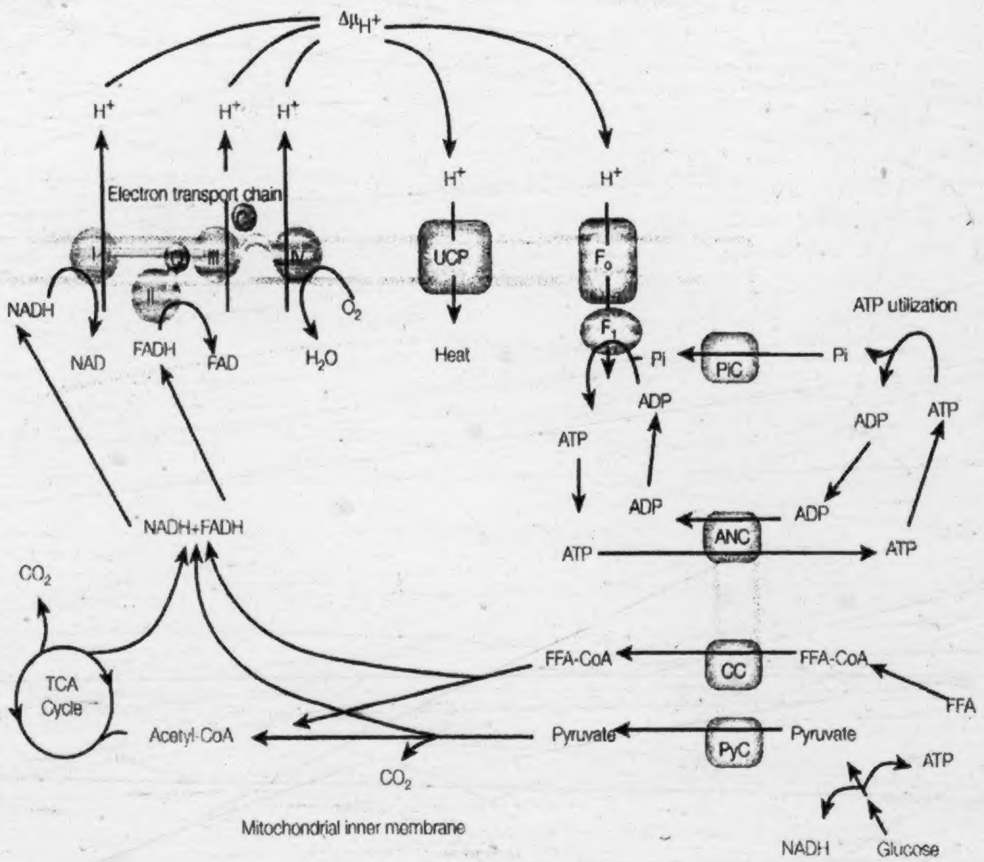
ΔμH+
H+
H+
H+
Electron transport chain
I
Q
III
C
IV
II
NADH
NAD
FADH
FAD
O2
H2O
H+
UCP
Heat
H+
F0
F1
ATP utilization
Pi
PiC
Pi
ADP
ATP
ADP
ATP
ATP
ADP
ANC
ADP
ATP
ATP
NADH+FADH
CO2
TCA Cycle
FFA-CoA
CC
FFA-CoA
FFA
Acetyl-CoA
Pyruvate
PyC
Pyruvate
ATP
CO2
Mitochondrial inner membrane
NADH
Glucose

Figure 2-3 Role of mitochondria in metabolism

Though the above figure simplistically represents the uncoupling protein activity as the transfer of protons into the mitochondrial matrix, dissipating the proton motive force generated in the intermembrane space, there are several models proposed for how this occurs. Some of the proposed models are well represented by a figure from a review by Nedergaard et. al. (2001), which further emphasizes the hypothesis that there is more than one carrier function associated with UCP-1, including those which are fatty acid dependent and those that are not. It is further possible, as has been suggested by several groups including

Klingenberg, Garlid and Skulachev, that fatty acids themselves can act as uncouplers through flip-flop proton transfer and detergent effects (in high concentration; Pressman and Lardy, 1956) in addition to their specific capacity to increase UCP-mediated uncoupling.

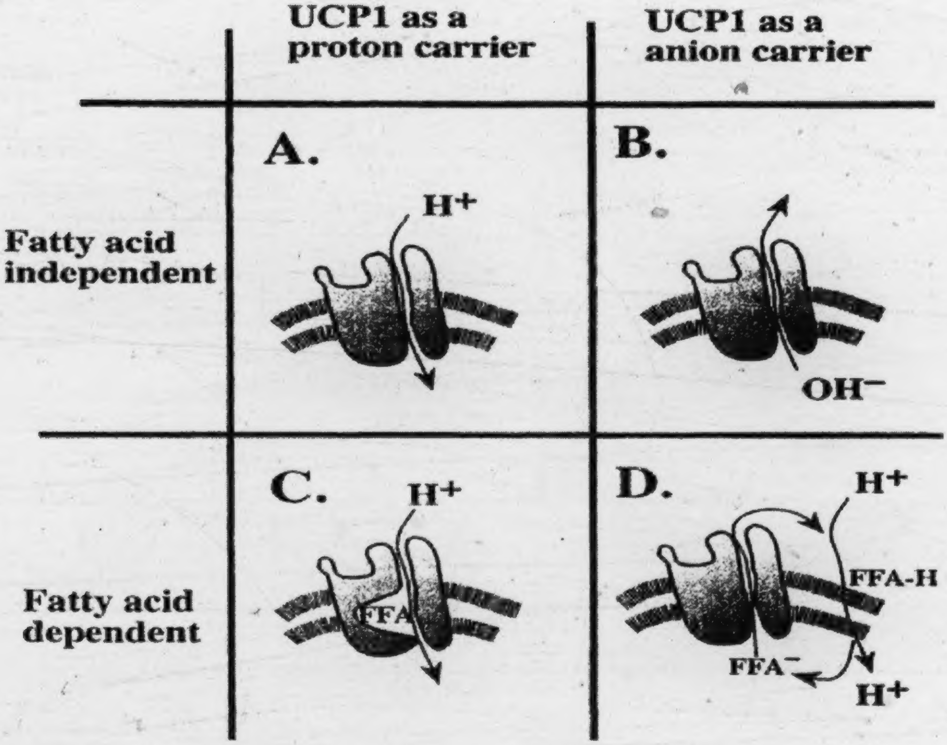
UCP1 as a proton carrier
UCP1 as a anion carrier
Fatty acid independent
Fatty acid dependent
A.
H+
B.
OH−
C.
H+
FFA
D.
H+
FFA-H
FFA−
H+

Figure 2-4 Proposed models of UCP-1 proton transport

Part A of the above figure represents the capacity of UCP-1 to directly uncouple via proton transport, this is generally accepted as is the concept that this is inhibited by purine nucleotide binding. Part B of the above figure is included to represent a non-specific capacity for UCP-mediated anion transport. Much less work has been dedicated to anion transport by UCP, though more may appear to support the hypotheses that UCP homologues are involved in compensating the accumulation of reactive oxygen species in the mitochondrial matrix (which is perhaps more relevant to UCP-2 and UCP-3). The last two components of Nedergaard's figure illustrate the two schools of thought with regards to free fatty acid involvement in uncoupling. More evidence tends to support the model illustrated in part D than that of a direct fatty acid-binding regulation of UCP as shown in part C but both systems may be involved. It is known that other proton transport mechanisms capitalize on the carboxyl unit of fatty acids to carry the protons, but few examples of fatty acid steric binding to carriers have been verified in other physiological systems. Additionally, steric binding would likely favour fatty acids of some particular size or characteristics, but all free fatty acids utilized have resulted in nearly the same quantitative effect.

2.1.1.5 Regulation of UCP gene expression

As previously mentioned (in the review of sympathetic activation of brown adipose), the expression of UCP-1 in brown adipose is influenced by sympathetic signaling, and it is known that this effect is a function of the well-characterized adrenergic receptor-G-protein coupled increase in cAMP. The elevation in cAMP activates protein kinase, which phosphorylates (serine/threonine) cyclic AMP-responsive element binding protein (CREB). CREB acts directly through a cis CRE enhancer site, upstream of the UCP gene, to increase transcription of mRNA. More uncoupling protein is thus translated and inserted into the mitochondrial inner membrane. The following figure from

Nedergaard's review (2001) illustrates this regulation that is so important to the sympathetic regulation of brown adipose non-shivering thermogenesis.

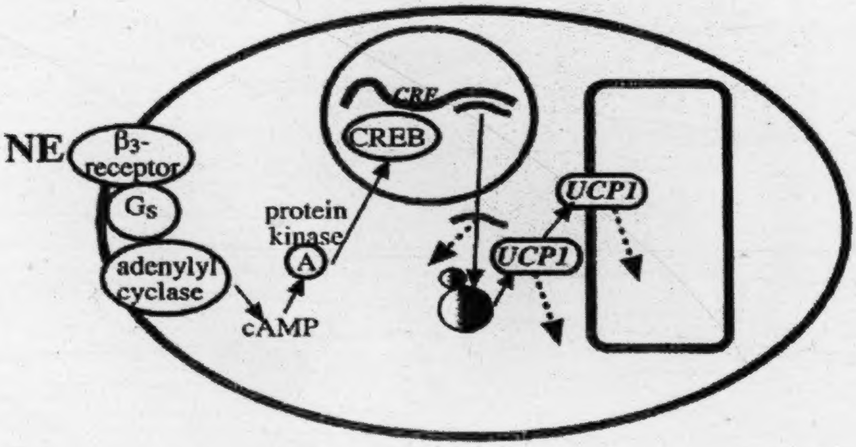

Figure 2-5 Sympathetic up-regulation of UCP-1

It was also mentioned earlier that thyroid hormone can influence the UCP mRNA by increasing its half-life. It should also be noted that synergistic activation by NE and triiodothyronine (T3) results in increased transcription of UCP-1 gene in primary cultures of brown adipocytes (Obregon et. al., 1996). The action of thyroid hormones on UCP gene expression involve the binding of thyroid hormone receptors (THR) at thyroid hormone response elements (TRE) which are present in an activator region upstream of the UCP gene. By performing and evaluating a series of deletions in the vicinity of the TRE, using electrophoretic mobile shift assays (EMSA) and DNA-footprinting, it was discovered that a 39 bp sequence downstream of the TRE was essential for the synergism between the CRE and TRE activation sites (Rabelo et. al., 1996). All of these activators show binding affinity in the "activator element" located between –2490 bp and -2280 bp of the rat UCP gene (Cassard-Doulcier et. al., 1998), this

211 base pair region was validated when a homologous 220 bp activator element was identified in the mouse UCP1 gene (-2530 bp to –2310 bp) (Kozak et. al., 1994). A figure that diagrammatically represents the rat UCP gene activator element is borrowed from a review by Silva and Rabelo (1997).

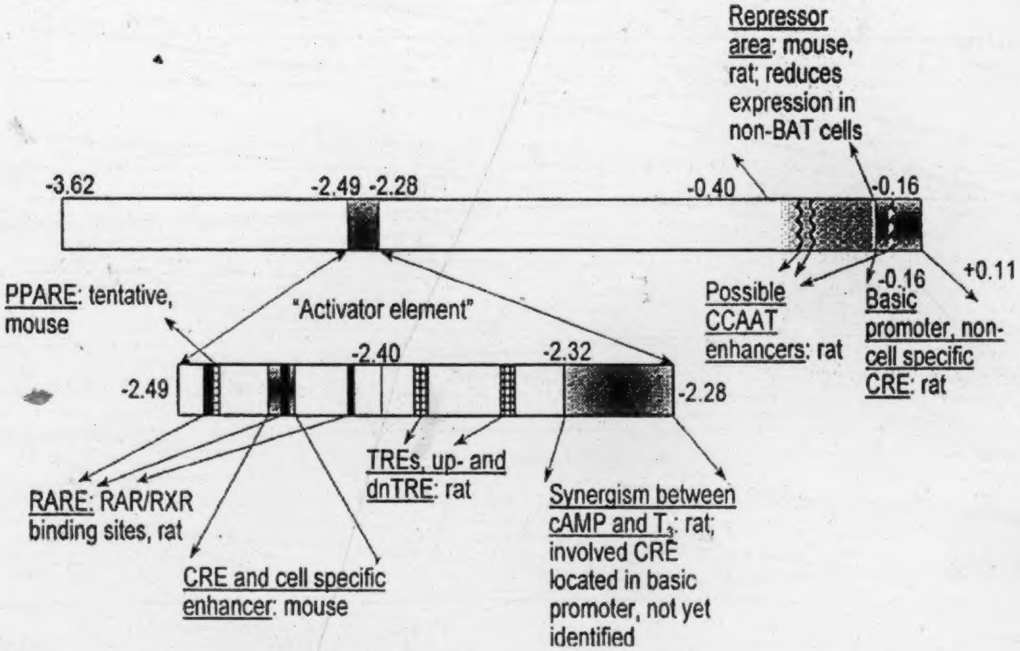
Repressor area: mouse, rat; reduces expression in non-BAT cells
-3.62
-2.49 -2.28
-0.40
-0.16
+0.11
-0.16
PPARE: tentative, mouse
"Activator element"
Possible CCAAT enhancers: rat
Basic promoter, non-cell specific CRE: rat
-2.40
-2.32
-2.49
-2.28
TREs, up- and dnTRE: rat
RARE: RAR/RXR binding sites, rat
Synergism between cAMP and T3: rat; involved CRE located in basic promoter, not yet identified
CRE and cell specific enhancer: mouse

Figure 2-6 Representation of UCP-1 gene activator element

As is apparent from the above diagram of the rat UCP gene activator element, there are other classes of activators that have binding sites upstream of the UCP gene and this includes the peroxisome-proliferator activated receptors (PPARs). There are three known PPARs in rat, of which PPARα and PPARγ are relevant to UCP gene activation, lipid transport and metabolism, and adipocyte differentiation (Kersten et. al., 2000). PPARs function in a similar fashion as other hormone receptors, binding to their specific sequence element in the promoter region of target genes, but it is also known that these PPARs function as heterodimers with another hormone receptor, RXR (a receptor for retinoic acid, retinoid X receptor). Abnormalities in PPAR function or expression level, have been implicated in many chronic diseases including diabetes, atherosclerosis, obesity and cancers (Kersten et. al., 2000). PPARγ ligands (thiazoladinediones) are utilized as treatments for type II diabetes, such as troglitazone and rosiglitazone, so their role in metabolic regulation is well substantiated. Natural intrinsic ligands for the PPARs include prostaglandins, fatty acids such as linolate, and conjugated linolenate (Moya-Camarena et. al., 1999). PPARs have been found to activate the expression of UCP and they function as a heterodimer with the RXR receptor, each binding to its individual specific promoter binding motif (Medvedev et. al., 2001). The binding of PPARs to their promoter sequence motif requires the double E-box motif in order to induce UCP gene expression activation (Medvedev et. al., 2000).

The other class of hormone receptors with a binding motif in the UCP gene promoter are the retinoid-activated receptors, these receptors also function as RXR heterodimers (Rial et. al., 1999). It is of interest that this class of receptors, capable of inducing UCP gene expression, can be activated by vitamin A (retinoic acid) derivatives and also that some fatty acids like linoleic acid can act as ligands for these receptors. Thus, at least part of the observed increase in mitochondrial uncoupling observed in the presence of higher free fatty acids may involve the induction of the retinoid-activated receptors promoter sequence in the UCP gene.

It is apparently an accepted practice in Japan to restrict cattle access to vitamin A, sometimes until they are so deficient in this vitamin that they become blind (E. Okine, personal communication). This practice was implemented as an outcome of research showing that vitamin A was negatively correlated with quality of beef and its adiposity since the palatability associated with high marbling adiposity in their beef is a market driven in Japan (Oka et. al., 1998). It is possible that these vitamin A-deficient animals may express substantially less uncoupling proteins and thereby become more energetically efficient and retain more energy as body fat.

It is evident at this point that there are many classes of molecules capable of activating specific sequence motifs in the UCP gene promoter region, which hints at the complexity of the in vivo regulatory mechanisms that mediate its physiological activity in mitochondria. Furthermore, the previous figure also indicates the presence of an as yet uncharacterized "repressor area" upstream of the UCP gene, which will also be important in the endeavour of a better understanding of UCP regulation and function.

There seems unquestionable evidence for an important role of UCP-1 in brown fat non-shivering thermogenesis, in newborn animals and hibernating species, but the distribution of UCP-1 appears to be limited to this tissue type. Though I have detected small amounts of UCP-1 can be detected in samples of other bovine white adipose depots, one cannot preclude the possibility that this represents brown adipocyte contamination (Murdoch et al., unpublished data). The UCP-1 gene is well characterized as being under the adaptive influence of sympathetic activation which further emphasizes its significance in cold-induced adaptive thermogenesis. It is useful at this point to briefly mention the apparent synergistic regulation of two other genes that appear relevant to this thermogenic pathway in brown adipose mitochondria. A recent study by Salvatore (1998) reported the rapid increase in expression of the guanosine monophospate reductase gene in cold-exposed brown adipose tissue. This becomes significant if one considers that the function of this gene is to convert guanosine nucleotides to IMP nucleosides, extending the hypothesis that cold-induced activation of this gene could effectively reduce the innate inhibition of UCP-1 by GDP nucleotides. A second gene regulated by cold in brown adipose adds to the complexity of regulatory aspects of UCP-1-mediated thermogenesis, this gene is for a thermogenic coactivator; PGC-1. It was discovered that PGC-1 mRNA expression is greatly elevated upon cold exposure in both mouse brown fat and skeletal muscle (Puigserver et. al., 1998). Moreover, PGC-1 vastly increases the transcriptional activity of both PPARγ and thyroid hormone receptor on the promoter of UCP-1 (Wu et. al., 1999). PGC-1 also activates an important mitochondrial transcription factor (mtTFA) that is a known direct regulator of mitochondrial DNA replication/transcription and as such positively influences the expression of genes such as cytochrome oxidase, a key enzyme in the electron transport chain (Wu et. al., 1999). The citations presented thus far in the literature review are evidence for the importance of utilizing molecular and physiological approaches towards characterizing and understanding the complex regulatory mechanisms involved in the control of a single tissue's metabolic state. This does not even address attempts to understand metabolic regulation in the whole animal. The following section will include a review of another candidate gene, uncoupling protein-2.

2.1.2 Uncoupling Protein-2

With the discovery of UCP-1 and the identification of some of its metabolically significant attributes it became the pursuit of many researchers to identify homologous proteins that may be expressed in tissues other than brown adipose. This goal was realized in 1997 when screening of a mouse skeletal muscle and lung cDNA library, using an UCP-1-derived probe, identified a clone with considerable sequence homology to that of UCP-1 (Fleury et. al., 1997; Gimeno et. al., 1997). It was shown that this gene had 56% homology with the UCP-1 gene and protein sequence homology at the level of 59%. The dramatic, and important, difference between UCP-1 and UCP-2 is the ubiquitous expression of UCP-2 in many different tissue types including brain, white adipose, and skeletal muscle. This broad distribution, along with its similarity to UCP-1, rendered UCP-2 an attractive candidate for the regulation of metabolism, body weight and composition in large animals including humans and cattle. UCP-2 has been shown to retain the capacity to uncouple ATP synthesis from electron transport, when expressed in vitro in yeast and other mammalian cells (Fleury et. al., 1997; Ricquier and Bouillaud, 2000). Furthermore, a study on 640 French Canadians predisposed to weight gain reported linkage between three microsatellite markers that encompass the UCP-2 gene location (in humans 11q13), with resting metabolic rate, body mass, percentage body fat, and overall fat mass (Bouchard et. al., 1997). In a recent review by Langhans (2002), he alludes to the discovery that, in tumour-bearing humans that have entered a state of cachectic hypermetabolism, an observed increase in skeletal muscle UCP-2 and UCP-3 contribute to elevated resting energy expenditure in these individuals. When such information came to light, it became important to include this gene as a candidate in our bovine efficiency study, and further to examine the comparative expression of this gene in bovine tissues.

2.1.2.1 Characteristics of UCP-2 (UCPH, Gimeno et. al., 1997)

Similar to other members of the inner mitochondrial membrane protein family, UCP-2 is roughly 300 amino acids long, and is tripartite with three comparable 100 a.a. domains. Unlike UCP-1, UCP-2 has eight exons and the two extra exons are responsible for encoding the longer 5' untranslated region while the other six exons each encode a transmembrane domain. The overall size of the UCP-2 gene is approximately 7 Kb. It is not found on bovine chromosome 17 like UCP-1 but rather mapped to bovine chromosome 15 (chromosome 11 in humans, 7 in mice). As is the case with UCP-1, there is no post-translational processing of the protein, but several amino terminal residues are conserved and speculated to be important for the mitochondrial inner membrane targeting and insertion.

2.1.2.2 UCP-2 Tissue Expression, Activity and Regulation

Rapid progress in terms of evaluation of UCP-2 can be attributed to the utilization of much of what was already established for UCP-1, and in effect studies designed to compare these two homologues. This led to the rapid characterization and mapping of the eight exons, start, stop, and poly-adenylation signals within the UCP-2 gene (Yamada et. al., 1998; Pecqueur et. al., 1999). The following figure borrowed from a paper by Tu et. al. (1999) outlines these aspects of the human UCP-2 gene.

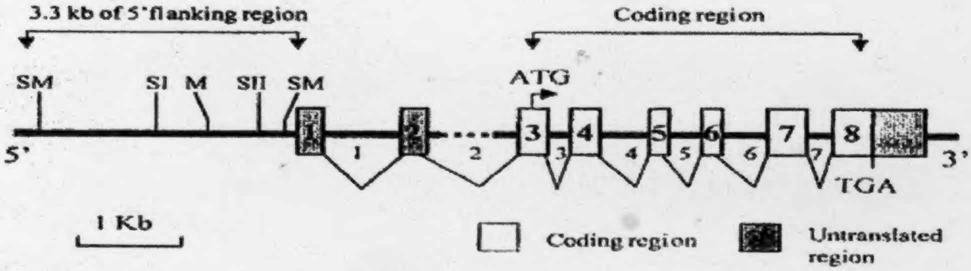

Figure 2-7 Human UCP-2 gene structural organization

It was shown that UCP-2 gene expression in the white adipose of mice was elevated by treatment with PPARα and PPARγ agonists as had been observed for UCP-1 (Aubert et. al., 1997). The PPAR ligands also stimulated UCP-2 mRNA expression in rat skeletal muscle (Fabris et. al., 2001) and this could be further elevated by coadministration of activators of the RXR receptor; rexinoids (Emilsson et. al., 2000). The observed effect of PPAR agonists on UCP-2 gene expression, was similar to that observed for UCP-1, in that it required activated PPAR interaction with a double E-box sequence motif and this interaction was potentiated by coordinated binding, in the form of a heterodimer, with activated RXR (Medvedev et. al., 2001).

Several other reports provided evidence that lipids could influence the expression of UCP-2 gene in several tissues. For instance, in an attempt to ascertain if induction of UCP-2 in white adipose was different for obesity-resistant and obesity-prone mice, Surwit et. al. (1998) fed a high fat diet to two strains of mice, one obesity-prone the other obesity-resistant, for four weeks and evaluated their respective UCP-2 mRNA levels. They found that the white adipose expression in the obesity-resistant mice were elevated 2.7 fold while they remained unchanged in the obesity-prone mice (Surwit et. al., 1998). This result suggested the possibility that up-regulation of uncoupling protein-2 in adipose may function as a compensatory means of preventing obesity due to consumption of a high fat diet. This high dietary fat-induced UCP-2 gene expression was not corroborated in an eight week study, and examination of skeletal muscle and white adipose UCP-2 mRNA expression levels (Gong et. al., 1999). The latter investigators suggested that the discrepancy between the two studies may be related to the fairly small observed changes, the different time period, or perhaps individual whole animal differences. An examination of human subcutaneous fat in subjects infused with lipids, relative to saline-infused control subjects, found an increase in mRNA for several genes including; UCP-2 and PPARγ (Khalfallah et. al., 2000; Nisoli et. al., 2000) Thus, *in vivo* gene expression of UCP-2 in subcutaneous adipose is affected by circulating lipids. Examination of rat muscle biopsies also indicated an increase in UCP2 mRNA expression in skeletal muscle before and after lipid infusion (Khalfallah et. al., 2000). As well, a primary cell culture of human muscle cells exposed to ω-6-polyunsaturated fatty acid showed a three fold increase in UCP-2 mRNA expression (Chevillotte et. al., 2001). This group supplied evidence that the response to this particular fatty acid involved both PKA and PPARβ pathways of gene induction. Increases in UCP-2 gene expression were further reported to be induced by dietary fish oil in rat small intestine (Murase et. al., 2001), fatty acids in rat neonatal cardiomyocytes (Van der Lee et. al., 2000) and dietary linoleic acid in human skeletal muscle (Ryder et. al., 2001). Using recent micro-array technology, the impact of a high fat diet on gene transcripts related to mitochondrial function were characterized and revealed increased expression of UCP-2 and cytochrome c oxidase subunit III, and further that these were not influenced by antioxidant supplementation (Sreekumar et. al., 2002).

It has been suggested, and appears probable, that lipids do influence the expression of uncoupling proteins in various tissues, and that this is likely mediated through the activation of PPAR isoforms and RXR hormone receptors that have direct and synergistic effects on promoter regions of UCP genes.

These results not only support the potential role of UCP-2 in nutrient-induced metabolic adaptation but are also consistent with hypotheses that UCP-2 may play an integral role in prevention of chronic illness such as type II diabetes, whole animal hyperlipidemia and obesity. Furthermore individual variability in both the ability to respond and the magnitude of the response in terms of increased UCP-2 expression remain popular components in theories to explain the variability observed regarding propensity for fat deposition in both animals and humans. This theory is well supported by a recent study involving dietary α-linolenic acid-rich diacylglycerols. It was found that mice fed diets high in α-linolenic diacylglycerol had elevated UCP-2 mRNA and β-oxidation levels in their small intestine and displayed significantly reduced body weight gain in association with slightly higher rectal temperatures, as compared to mice fed diets with lipid content comprised of saturated triacyglycerols (Murase et al, 2002).

Transcriptional regulation of UCP-2 gene in brown adipose is also influenced by retinoic acid, again similar to UCP-1 (Rial and Gonzalez-Barrasso, 2001; Scarpace et. al., 2000). This is attributed to activation of RXR, RAR and PPAR classes of intracellular hormone receptors.

Cardiac muscle expression of UCP-2 was increased in rats exposed to both T3 and T4, which supports the concept that this uncoupler may be important in whole animal metabolic rate (Lanni et. al., 1997). Rat skeletal muscle and liver UCP-2 gene expression were also elevated in T3 treated mice (Jekabsons et. al., 1999). Moreover, this increase was shown to be positively correlated with the increase in measured resting metabolic rate (Jekabsons et. al., 1999) All of these factors point to a similarity between the gene promoters for UCP-1 and UCP-2. The subsequent figure illustrates the similarities between UCP-1 and UCP-2 in their promoter regions with similar hormone receptor binding sequence motifs present upstream of the human UCP-2 gene. Some small differences between these two homologues promoter regions include the lack of classical promoter TATA and CAAT boxes but rather a GC rich sequence (-65bp) that includes adipose specific promoter sites (AP1/AP2) and a Sp1 sequence motif (Tu et. al., 1999).

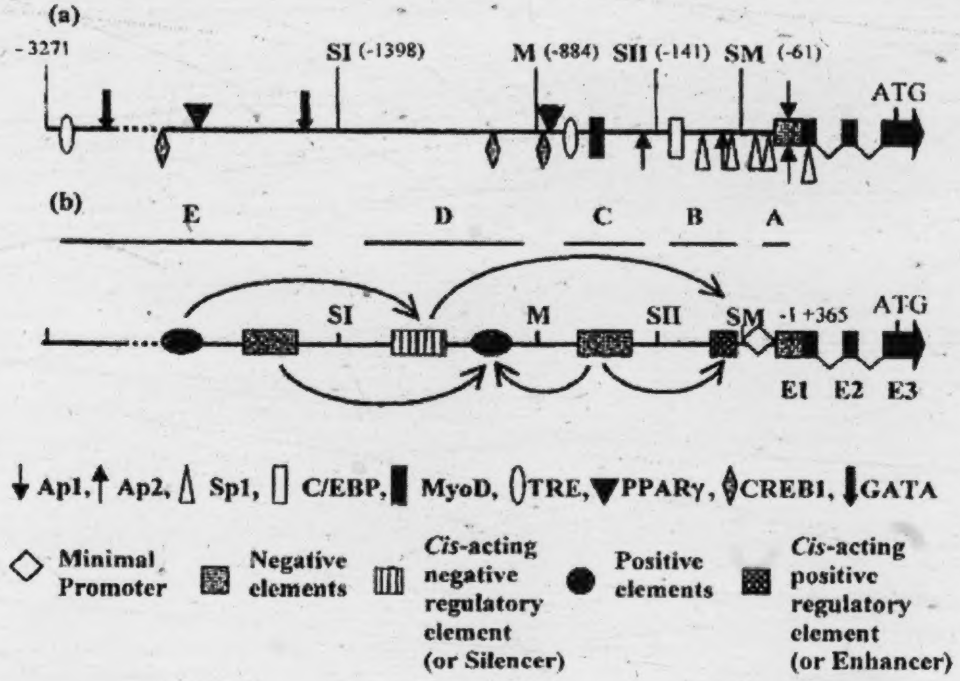
(a)
- 3271
SI (-1398)
M (-884)
SII (-141)
SM (-61)
ATG
(b)
E
D
C
B
A
SI
M
SII
SM
-1 +365
ATG
E1
E2
E3
Ap1, Ap2, Sp1, C/EBP, MyoD, TRE, PPARγ, CREB1, GATA
Minimal Promoter
Negative elements
Cis-acting negative regulatory element (or Silencer)
Positive elements
Cis-acting positive regulatory element (or Enhancer)

Figure 2-8 UCP-2 up-stream promoter regions restriction sites within promoter region.
Restriction digestion products (top), promoter binding element domains (bottom)

Also apparent is the presence of a CREB sequence motif within the UCP-2 promoter region, this indicates the potential for sympathetic cascade activation of the cAMP pathway and regulation of UCP-2 gene expression. The expression of UCP-2 in many different tissue types also means that there are different sympathetic innervation and adrenergic receptors present in these tissues and thus the effect of NE activation becomes quite tissue-specific. Fleury et. al. (1997) did show that β-adrenergic signaling resulted in increased UCP-2 mRNA expression in WAT. Of additional interest, is the presence of cis-acting negative regulatory domains (silencers) within the UCP-2 promoter which may explain some of the varied expression in different tissues though this sequence motif and receptors that influence it have yet to be characterized.

The presence of a MyoD binding site is also of importance, since this is a muscle specific gene activator especially relevant in muscle differentiation and fibre deposition. This may suggest that it also influences UCP-2 expression and thus may be important in the embryonic development of muscle or it may also modulate the expression of UCP-2 in adult animals that are showing muscle hypertrophy or hyperplasia. This property may be relevant when examining the effect of training scenarios, which can also induce hypertrophy. The last characteristic of the UCP-2 gene promoter region to be discussed is related to a hypothesized role of UCP-2 in an important immune response; fever. The outer coat of several types of pathogenic bacteria express lipopolysaccharide (LPS). It has long been known that when LPS moieties are introduced in the blood of mammals they are pyrogenic, that is, they elevate body temperature. It has been shown that LPS treatment increases the release of the cytokine, Tumour necrosis factor-α (TNF-α). Several recent reports have shown that activation of TNF-α results in increased UCP-2 expression in rat, BAT, WAT, and skeletal muscle (Masaki et. al., 1999) as well as rat hepatocytes (Cortez-Pinto et. al., 1998). Cortez-Pinto was the first to hypothesize that this increase in UCP-2 expression may be a key aspect of the pyrogenic effects of bacterial LPS and may suggest an important and systemic role for UCP-2 in the immune response related to fever. Further support for this was generated by the discovery that LPS exposure also activated PGC-1, an activator of PPARs, and that the increases in skeletal muscle

UCP-2 (and UCP-3) were proportional to increased measures of hyperthermia and mitochondrial proton leak kinetics (Yu et. al., 2000).

Additional support for a role of UCP-2 in adaptive thermogenesis is conveyed by its consistent upregulation in response to cold observed in a broad range of studies, including those in chickens (Raimbault et. al., 2001), hummingbirds (Vianna et. al., 2001), and even a UCP homologue in plants (Pecquer et. al., 2001).

There also appears to be a nutritional regulation of UCP-2 expression, since lipids can activate the UCP-2 gene transcription. It has been reported that isoenergetic substitution of carbohydrate with fat resulted in increased expression in rat muscle, though thermogenesis was lower in the high fat than the low fat diet animals (Samec et. al., 1998). UCP-2 is positively correlated with plasma insulin levels, except in individuals with non-insulin-dependent diabetes mellitus (NIDDM) (Vidal et. al., 1999). Uncoupling protein-2 is consistently elevated in skeletal and white adipose of acute-fasted rats (Pinkney et. al., 2000) and humans (Millet et. al., 1997) and also when hypophagia is induced as in MAC-16-induced cancer cachexia (Bing et. al., 2000). However this relationship is again abnormal in skeletal muscle of type-2 diabetics, where UCP-2 mRNA and protein are unchanged by acute fasting. The story becomes more complex when it is considered that UCP-2 expression in white adipose is increased by chronic leptin treatment, but down-regulated during acute leptin treatment (Combatsiaris and Charron, 1999). However, this still supports the idea that reduced UCP-2 expression may be a maladaptive response to sustained energy surplus and that this may be relevant to the onset and maintenance of obesity and obesity-related disease such as diabetes.

There is one further hypothesis with regards to the systemic role of UCP-2 expression, and that is that it functions as a moderator for the production of reactive oxygen species (ROS). The reduction of mitochondrial hydrogen peroxide generation with increased UCP-2 expression has been reported (Negre-Salvayre, 1997). During states of excess lipid oxidation it has been shown that there is an increase in production of ROS, including peroxide (Berson et. al., 1998). Since the accumulation of ROS products can become toxic due to undesirable oxidation of enzymes, it is hypothesized that under conditions where the mitochondria are predisposed to increased ROS production increased UCP expression could partially moderate this accumulation. As mentioned, under conditions of fasting UCP-2 is up-regulated, as would be the rates of lipolysis, this increase in UCP is in contrast to what would be expected if UCP's sole function were as a positive regulator of thermogenesis. Instead, it is believed that UCP can attenuate the ROS production under scenarios of increased lipolysis by offering an alternative route for protons to reenter the mitochondrial matrix and neutralize the ROS accumulating there. It has further been suggested that the propensity to generate ROS is also high in skeletal muscle in fasted animals (or in animals enduring high rates of activity) and that the expression of UCP is elevated in these scenarios and may function in a ROS compensatory role. Another extension of this hypothesis is that the different populations of skeletal muscle mitochondria (subsarcolemmal and intermyofibrillar) may be different in their involvement in this function. It has been shown that a large degree of uncoupling of subsarcolemmal mitochondria appears to be generated in these scenarios, even though the UCP expression appears to be similar in these two mitochondrial subpopulations (Iossa et. al., 2001). The generation of an UCP-2 knockout mouse has added dramatic support for this hypothesized function of UCP-2. Arsenijevic et. al. (2000), found that UCP-2 deficient mice were resistant to toxic shock associated with *Toxoplasma gondii* infection. The explanation for this is that UCP-2 interferes with the generation of ROS in macrophages and that this turns out to be detrimental to the wild-type animals because ROS confer a protective aspect against *T. gondii* infection. Thus, the higher ROS in UCP-2 knockout mice attenuates the pathogenicity of this particular infection (Arsenijevic et. al., 2000). The next figure, is a simplified illustration of how UCP may reduce the generation of reactive oxygen species (Vidal-Puig, 2000).

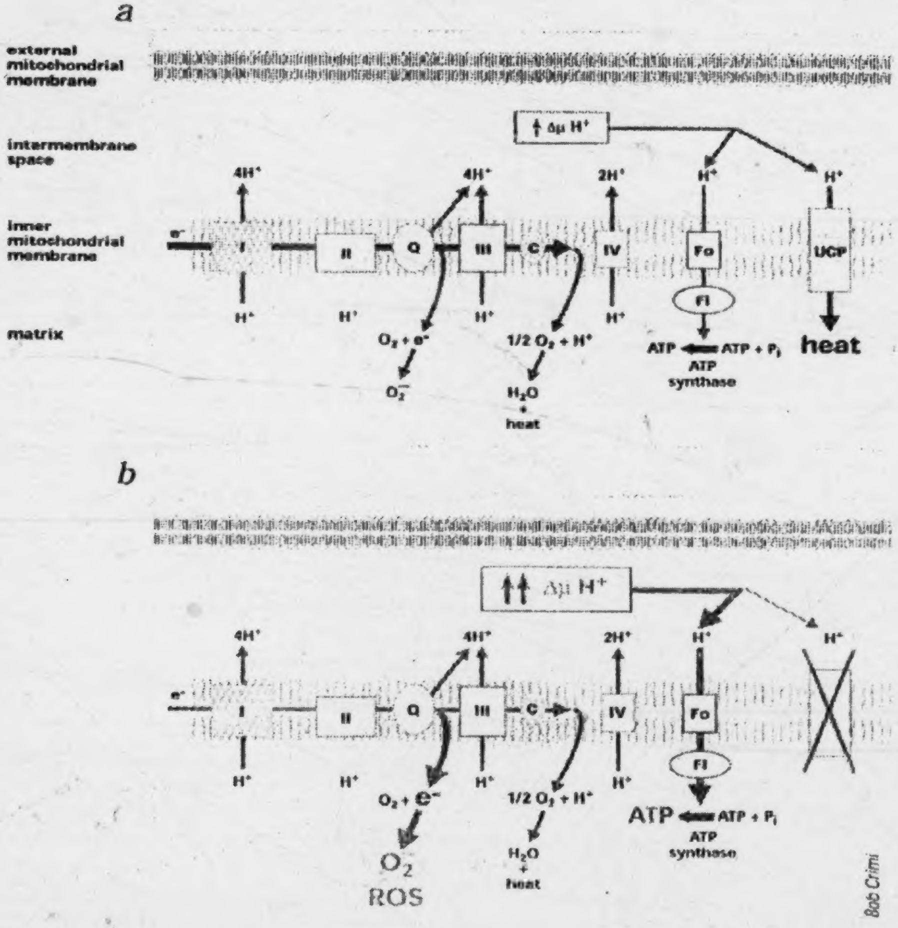
a
external mitochondrial membrane
intermembrane space
inner mitochondrial membrane
matrix
↑ Δμ H⁺
4H⁺
4H⁺
2H⁺
H⁺
H⁺
e⁻
I
II
Q
III
C
IV
Fo
UCP
Fl
H⁺
H⁺
H⁺
H⁺
O₂ + e⁻
1/2 O₂ + H⁺
O₂⁻
H₂O + heat
ATP ATP + Pᵢ
ATP synthase
heat
b
↑↑ Δμ H⁺
4H⁺
4H⁺
2H⁺
H⁺
H⁺
e⁻
I
II
Q
III
C
IV
Fo
Fl
H⁺
H⁺
H⁺
H⁺
O₂ + e⁻
1/2 O₂ + H⁺
O₂⁻
ROS
H₂O + heat
ATP ATP + Pᵢ
ATP synthase
Bob Crimi

Figure 2-9 Diagram of mitochondrial establishment of proton electro-chemical gradient, and UCP uncoupling.
(a) UCP generation of heat via uncoupling of proton gradient from ATP synthesis
(b) Model representing increased ROS accumulation in the absence of uncoupling protein

As the recently developed UCP-2 knockout mice are utilized for additional experimentation a better grasp of the function of UCP-2 in various tissues will be acquired. It is already notable that the UCP-2 knockout mice are not phenotypically obese, nor do they appear to be dramatically cold-sensitive (Arsenijevic et. al., 2000). These attributes may be indicative that mitochondrial

UCP-2 is not responsible for mediating either of these physiological events independently though they may still participate in these adaptational metabolic processes. Since the development of the UCP-2 knockout mice and the discovery that the increased ROS production conferred resistance to *Toxoplasma gondii* , it has since been found that wild-type mice maintain some capacity to generate ROS in macrophages as the presence of LPS acts on a negative regulatory domain in intron 2 to suppress UCP-2 expression and allow for increased levels of ROS (Kizaki et al, 2002). This group further characterized this region of UCP-2 intron 2 as being the site of a NO enhancer element. They concluded that LPS-stimulated signals would suppress UCP-2 expression by partially interupting the functioning of the intronic enhancer allowing for up-regulation of the reactive oxygen species sufficient to mediate an antimicrobial attack. However since this is only a partial inactivation the homologous recombinant UCP-2 knockout mice have a more dramatic increase in ROS levels and hence are more effective at responding to T. *gondii* challenge.

The characterization of UCP-2 gene expression in bovine tissues is non-existent and thus assessment of its gene expression will be of interest even if it is shown that it is not a major regulator of whole animal metabolic efficiency.

2.1.3 Uncoupling Protein-3

Not long after the isolation of UCP-2 two separate groups reported the isolation of another uncoupling protein homologue, both were isolated from human skeletal muscle cDNA libraries. Both Boss et. al., (1997) and Vidal Puig et. al. (1997) isolated clones from the skeletal muscle cDNA libraries and reported homologies of 57% with UCP-1 and 73% with UCP-2. In fact Boss et. al. (1997) isolated two separate isoforms a short 275 aa (UCP3s) and a long isoform 312 aa (UCP3{L}). The short isoform appears to be a truncated version of the long form and has been reported in humans, mice and rats thus far. Although, UCP-3 shows very high levels of homology with UCP-2, its tissue-specific expression is much less ubiquitous than UCP-2. It appears that UCP-3 is mainly an isoform related to striated muscle, although trace amounts have been indicated in both WAT and BAT. It was reported that UCP-3 was present in both skeletal and cardiac muscle (Boss et. al., 1997). Furthermore, in a study of 640 French Canadians predisposed to weight gain it was reported that there was a significant linkage between three microsatellite markers that encompass the UCP-2 and UCP-3 gene location (in humans 11q13), with resting metabolic rate, body mass index, percentage body fat, and overall fat mass (Bouchard et. al., 1997). This finding was corroborated in a study of PIMA Indians, also predisposed to obesity and type II diabetes, where a negative correlation between skeletal muscle UCP-3 and both RMR and BMI (Schrauwen et. al., 1999). However, when a less extreme population (in terms of propensity for morbid obesity) was examined, no difference was found between skeletal muscle UCP-3 mRNA expression in obese and lean caucasians (Millet et. al. 1997).

2.1.3.1 Characteristics of UCP-3

As would be expected by the high degree of homology with UCP-2, UCP-3 maintains the familial pattern of tripartite structure, with each domain being roughly 300 amino acids in length. The UCP-3 gene contains seven exons, one for each of the six transmembrane domains and a seventh representing a 5' untranslated domain. The short isoform described by Boss et. al. (1997) is missing the last transmembrane domain of the protein, and recall that this region is believed to be important for nucleotide binding in UCP-1. The UCP-3 gene has been mapped and is found to be in very close proximity and upstream of the UCP-2 gene in mice, humans, rats and cattle. The UCP-3 gene is located in cattle on chromosome 15, and is less than 100 Kb upstream of UCP-2 gene, and likely even closer than this conservative estimate. It is known that the UCP-3 and UCP-2 genes are separated by less than 7 Kb in humans and just over 8 Kb in mice, as is illustrated in the subsequent figures (Pecqueur et. al., 1999 top; Gong et. al., 1999 bottom).

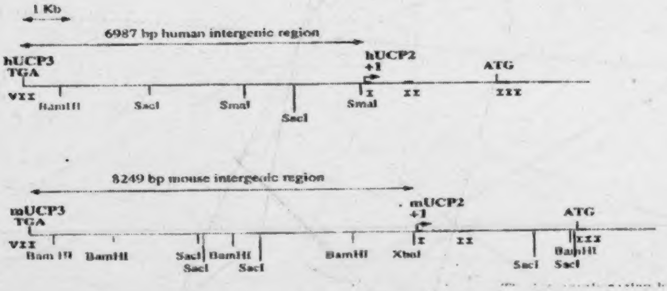
1 Kb
6987 bp human intergenic region
hUCP3
TGA
hUCP2
+1
ATG
VII
BamHI
SacI
SmaI
SacI
SmaI
I
II
III
8249 bp mouse intergenic region
mUCP3
TGA
mUCP2
+1
ATG
VII
Bam HI
BamHI
SacI
SacI
BamHI
SacI
BamHI
XbaI
I
II
SacI
BamHI
SacI
III

Figure 2-10 Human and mouse intergenic region of UCP-3 and UCP-2

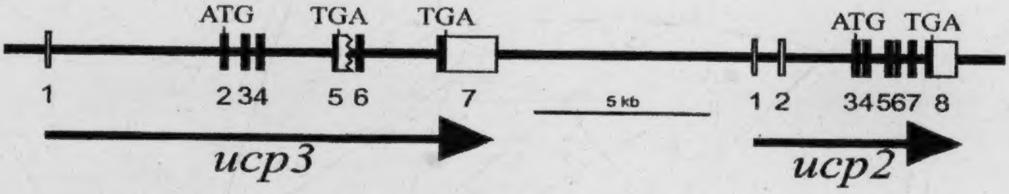
ATG
TGA
TGA
ATG
TGA
1
234
5 6
7
5 kb
1 2
34567 8
ucp3
ucp2

Figure 2-11 Murine UCP-3 and UCP-2 exon boundaries.

It has been suggested that the close proximity of these genes may facilitate coordinated regulation of expression of these genes in specific tissues and physiological conditions (Pecqueur et. al., 1999). It does imply that the linkage of this region to indices of obesity could implicate both genes as mediators of this trait, though more stringent linkage analyses may exclude one of these genes all together.

2.1.3.2 UCP-3 Activity and Regulation

As was the case with UCP-2, the progress in terms of characterizing the induction of UCP-3 gene expression was rapid given that the foundation for productive candidates was formulated by the groundwork surrounding UCP-1. For instance, in 1997 it was reported that cold-exposed rats did not show elevated levels of UCP-3 transcripts in either skeletal muscle or BAT, but 5 day treatments with T3 increased gene expression in both tissues (Larkin et. al., 1997). This was corroborated in the same year by a study demonstrating that hypothyroid rats expressed three-fold less and hyperthyroid rats expressed six-fold more UCP-3 in their skeletal muscle as compared to normal control (Gong et. al., 1997). Gong also demonstrated that treatment with β-3 agonists elevated UCP-3 transcription in muscle and over-expression in yeast reduced the mitochondrial membrane potential indicating its ability to uncouple (Gong et. al., 1997). A more recent study involving T3 treatment of mice found a good correlation between both UCP-2 and UCP-3 up-regulation in skeletal muscle and increased resting metabolic rate (Jekabsons et. al., 1999). Boss et. al. (1998) re-iterated that two days of cold exposure failed to elevate UCP-3 expression in rat skeletal muscle but that one week of feed restriction (50%) decreased UCP-3 by 81% while acute 24 hour fasting elevated UCP-3 transcripts six-fold (Boss et. al., 1998). In transfected myoblasts, Boss et. al., also validated the capacity for UCP-3 to uncouple myoblasts (1998). These results suggest that UCP-3 expression may be more important in the regulation of energy expenditure, body weight, metabolic rate and nutrient sensitive metabolic adaptation rather than cold-induced thermogenesis. Of course skeletal muscle inherently can capitalize on heat production associated with shivering thermogenesis, that is known to be activated upon cold exposure, which may be theorized to eliminate the need for NST in skeletal muscle during exposure to lowered ambient temperatures.

The generation of mice with selective skeletal muscle over-expression of human UCP-3 has proven useful for the phenotypic traits resultant from this abnormality, and these mice are both hyperphagic and lean (Clapham et. al., 2000). Using magnetic resonance imaging, they found that the adipose deposition in both visceral and subcutaneous depots were drastically reduced in these human UCP-3 transgenic mutants (Clapham et. al., 2000). These transgenic mice displayed 25% increased resting oxygen consumption and regardless of their 50% increase in dietary intake, their plasma triglycerides were normal suggesting an increase in fatty acid oxidation (Clapham et. al.., 2000). The flip-side of this can be observed through the generation of UCP-3 gene knockout mice (UCP-3KO). Surprisingly these UCP-3KO mice did not develop obesity, but their plasma triglyceride concentrations were elevated and their skeletal muscle mitochondria were more coupled and had higher measured levels of ROS compounds than controls (Vidal-Puig et. al., 2000). Furthermore the UCP-3KO mice had normal internal body temperatures and were not cold-sensitive (Vidal-Puig et. al., 2000). This group remain cautious emphasizing that readers should not yet interpret these findings conclusive as to the lack of involvement of UCP-3 in these activities, but that other genes or pathways may be compensating for any deficits attributable to the UCP-3KO mice (Vidal-Puig et. al., 2000).

It has been shown that high fat diets (Matsuda et. al., 1997; Sreekumar, 2002), and direct plasma lipid infusion (Nisoli et. al., 2000; Khalfallah et. al., 2000) up-regulate UCP-3. Additionally vitamin A derivatives (Scarpace et. al., 2000; Solanes et. al., 2000), acute fasting (Boss et. al.,1997: Millet et. al., 1997) or acute food restriction (Iossa et. al., 2001) can elevate skeletal muscle UCP-3 expression. Further investigation showed that treatment with nicotinic acid (an inhibitor of lipolytic activity that can dramatically reduce the plasma free fatty acid increases associated with fasting), results in prevention of the fasting-associated up-regulation of UCP-2 and UCP-3 in slow oxidative muscle (*soleus*) but not in fast glycolytic (*gastrocnemius*) nor fast oxidative-glycolytic (*tibialis anterior*) (Samec et. al., 1998). In obese humans there was a strong correlation between plasma free fatty acid concentrations and skeletal muscle UCP-3 levels but this correlation was not observed in lean individuals (Sbraccia et. al., 2002). This group extended their findings by hypothesizing that circulating free fatty acids act as the predominant regulator of skeletal muscle UCP-3 gene expression once levels are above a yet to be determined threshold level. Another recent report suggests that high fat-induced UCP-2 and UCP-3 upregulation is more pronounced in humans with higher proportions of type IIA fibres, which are better able to shift from glucose to lipid oxidation than type I muscle fibres (Schrauwen, et. al., 2001). Schrauwen remains a strong proponent of the theory that UCPs are important for their ability to reduce the accumulation of mitochondrial ROS species that tend to be elevated under high states of lipolytic activity. His results seem to support differential regulation of the UCP-3 in accordance with expected increases in lipolysis. .

Hormones with known capacities to regulate whole animal metabolism such as leptin (Liu et. al., 1997; Scarpace et. al. 2000) and insulin (Pedersen et. al., 2001) have also been confirmed to influence UCP-3 expression.

As well, a lack of UCP-3 mRNA induction in skeletal muscle of type II diabetics during fasting indicates an association between this chronic disease and an abnormal UCP-3 transcriptional response (Vidal et. al., 1999), which may be due to insulin insensitivity. Moreover there appears to be a chronic reduction in UCP-3 skeletal muscle expression in NIDDM patients (Krook et. al., 1998) and streptazoacin induced diabetic (type 1 diabetes) rats had elevated plasma free fatty acids which resulted in increased skeletal muscle UCP-3 expression (Kageyama et. al., 1998). These trends are quite similar to the observed regulation of UCP-2, and suggest similar hypothesized roles for UCP-3 such as ROS limitation and adaptation of skeletal muscle metabolism in relation to plasma fatty acid levels. We know that free fatty acids can activate the Retinoid (RAR and RXR) and PPAR hormone receptors and PPARα ligands (Fibrates) and PPARγ ligands (Thiazoladinediones {TZDs}) have both been shown to increase UCP-3 expression dose-dependently in skeletal muscle (Pedraza et. al., 2000) and cardiac muscle (Young et. al., 2001). The TZDs have even been used in the treatment of NIDDM, to reduce the increased plasma free fatty acids. In a study evaluating the retinoic acid induction of skeletal muscle UCP-3 it was shown that a key muscle specific gene promoter, MyoD, was intricately involved (Solanes et. al., 2000). MyoD is also activated by retinoic acid, and this further ties in with the vitamin A restriction that is currently practiced in Japan. If UCP-3 promotes fatty acid oxidation in the skeletal muscle by reducing vitamin A availability then producers can reduce the UCP-3 and effectively elevate the triglyceride levels in the skeletal muscle. The decrease in lipolysis coupled with the increase in triglyceride storage in muscle would mean that vitamin A restriction would result in increased marbling of the meat. MyoD is a regulatory factor essential to the differentiation program of muscle cells thus a requirement of the UCP-3 gene promoter for MyoD is likely responsible for the preferential expression of this gene in differentiated muscle cells. Moreover, and perhaps from a production sense more interestingly, since MyoD is involved this may be a potential mechanism by which we can capitalize on a muscle-specific promoter to allow for specific deposition of intramuscular fat. This would effectively allow reduction of waste fat (subcutaneous) in cattle and also pigs as a recent study has demonstrated that UCP-3 is also present and specific to skeletal muscle in pigs (Damon et. al., 2000). The following figure from Solanes et. al.(2000), illustrates MyoD's accessory role in UCP-3 gene activation with RAR-RXR heterodimers.

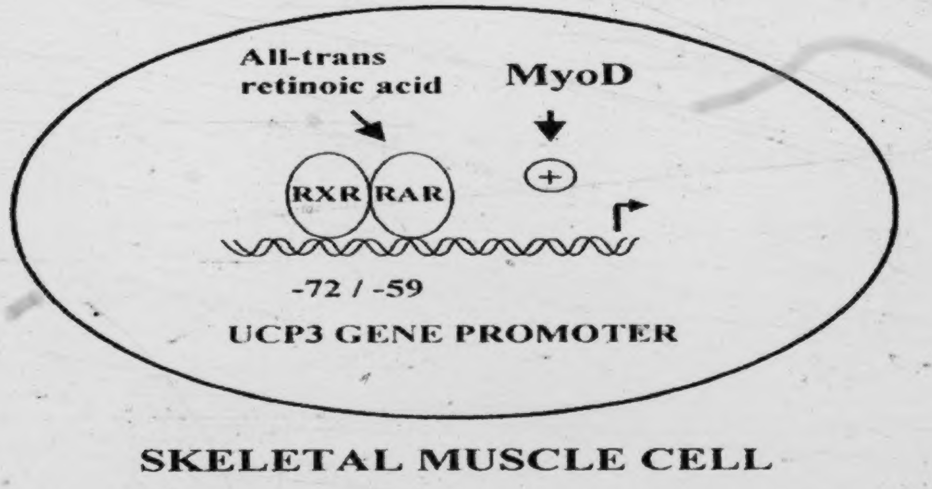

Figure 2-12 Schematic representation of UCP-3 gene promoter regulation by MyoD and retinoic acid.

As was observed for UCP-2 gene transcription, UCP-3 is also elevated by a TNF-α mediated response to bacterial lipopolysaccharides (LPS) and it may also be involved in the hyperthermic effect observed with exposure to these pyrogens, and the immune fever response.

Given the unique tissue distribution of UCP-3 primarily in skeletal muscle it has become of interest to determine the effect of muscle contraction and exercise on the expression of this gene. In an in vitro study by Pederson et. al. (2001), they found that 60-120 minutes after a modest 10 minutes of electrically-induced contraction there were consistent increases in skeletal muscle UCP-2 and UCP-3 expression. When comparisons are made in terms of fasting-induced UCP-3 expression between different fibre types there appear to be notable differences. Cortright et. al. (1999) was the first to report that acute exercise (2 hour treadmill run) elevated the UCP-3 mRNA expression levels in white (252%) and red (63%)

gastrocnemius, versus sedentary controls, but there were no significant changes in rats under going long-term exercise regimes (9 weeks of wheel running). Transcription rate of muscle specific UCP-3 gene was elevated 14-21 fold in white *gastrocnemius* (fast twitch glycolytic), 5-8 fold in red *gastrocnemius* (fast twitch, oxidative) and *plantaris* (mixed) but no change in *soleus* (slow-twitch oxidative) induced by fasting (Hildebrandt and Neufer, 2000). When Zhou et. al. (2000) evaluated UCP-3 expression in rat hindlimb muscle (*extensor digitorum longus*) they found that it was increased by acute exercise (30 minutes treadmill), hypoxia (90%Nitrogen-10% oxygen) and an activator of AMP kinase (AICAR). It was an unexpected finding that increased metabolic demand during fasting, mediated by two 2-hour bouts of treadmill exercise within the first 8 hours of a 24 hour fast, attenuated the fasting-induced transcriptional activation of skeletal muscle UCP-3 (Hildebrandt and Neufer, 2000). This result creates a bit of a paradox because all reports thus far have indicated that fasting and acute exercise should both elevate UCP-3, and the intent of this study was to determine if they were additive. These results in conjunction with the Samec et. al. study (1998) illustrates that there is a fibre-type specific expression and regulation pattern for UCP-3 in skeletal muscle. Furthermore it appears that fasting and exercise may have opposing mechanisms of UCP-3 regulation. To date, the available research has failed to clarify the mechanisms of this fibre type-selective regulation, and more investigation will be beneficial in determining the interplay between nutrient status and exercise on UCP-3 and UCP-2 expression. Two more recent studies have elucidated the link between activation of AMP-activated protein kinase and increased UCP-3 transcription in skeletal muscle of rats which will serve to clarify the influence of exercise on uncoupling gene expression (Stoppani et. al., 2002; and Putman et. al., in press). It is likely that UCP-3 expression is related to the metabolic recovery process in working skeletal muscle. Of course exercise is not a large consideration in livestock production but deposition of intramuscular adipose (marbling) may be influenced by variability in muscle fibre type and its relationship to uncoupling protein expression. Furthermore, although "thrifty" genotypes are detrimental to the health of persons in affluent societies, due to their promotion of obesity, much research has been directed in identification of polymorphisms in genes related to efficiency, and the agricultural directed research may be able to capitalize on such information upon evaluation in livestock. In a recent review of single nucleotide polymorphisms in human thrifty genes, UCP-2, UCP-3, leptin and leptin receptor (both to be reviewed subsequently) as well as several other genes were all identified as being related to elevated efficiency in human populations (Kagawa et. al., 2002).

It is apparent that uncoupling proteins are expressed in a variety of tissues, and are influenced by varied nutrient states, environmental conditions as well as in relation to some chronic diseases. The UCP's remain of great interest to research directed towards the physiological understanding of whole animal metabolism.

2.1.4 Leptin

The next group of candidate genes has a different context in terms of why it was selected for our energetic efficiency study. If one considers the following equation:

Metabolizable Energy (ME) = Retained Energy (RE) + heat production (HP)

$\therefore$ RE = ME –HP

What the above formula illustrates is that in order to influence the growth and deposition of tissues (RE) in livestock, while abiding by the first law of thermodynamics, one may only decrease HP and/or increase ME. The selection of the uncoupling protein gene candidates was initially based on their potential involvement in heat production via the uncoupling of the mitochondrial respiration from ATP synthesis. It was hypothesized that increased expression of these inner mitochondrial membrane carriers would be positively correlated to an animal's heat production. The next angle for maximizing livestock growth and efficiency is through maximizing metabolizable energy intake and the simplest manner by which to do this is by increasing nutrient intake (GE). The discovery of leptin, a satiety hormone established itself as a candidate for our study through its inferred capacity to affect the ingestion of nutrients (appetite) and in so doing an ability to reduce the total energy eaten.

It was long postulated that there must be a physiological regulator of nutrient intake and that this regulator must have the capacity to reflect the animals' nutrient and stored energy status. While working with phenotypically obese mice, the gene responsible for this obesity was identified in 1994 (Zhang et. al., 1994). This group established that this "ob" gene (in mice) encoded a 167 amino acid protein called "leptin" and further hypothesized that it would have a hypothalamic site of action (Zhang et. al., 1994). As a regulator of adiposity leptin quickly became a focus for it's potential involvement in the regulation of energy balance. Over the last several years it has become apparent that the leptin gene product is a critical participant in the homeostatic balance between triglyceride catabolism and storage.

2.1.4.1 Characteristics of Leptin

The leptin gene (Ob in mice) is located on chromosome 4 in cattle (chromosome 7 in humans), it is approximately 15 Kb, and contains 3 exons. The three exons encode a 4.5 Kb mRNA and translate into a 166 amino acid peptide. The first exon encodes a 5' untranslated region, and the last two encode the amino acid sequence. The next two figures illustrate the gene arrangement for the human OB exons and cDNA (Isse et. al., 1995) and the human Ob gene with some promoter characteristics (Gong et. al., 1996).

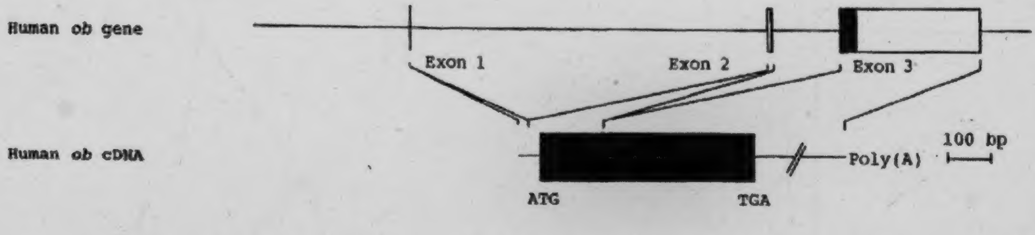

Figure 2-13 Exon organization of human Ob gene

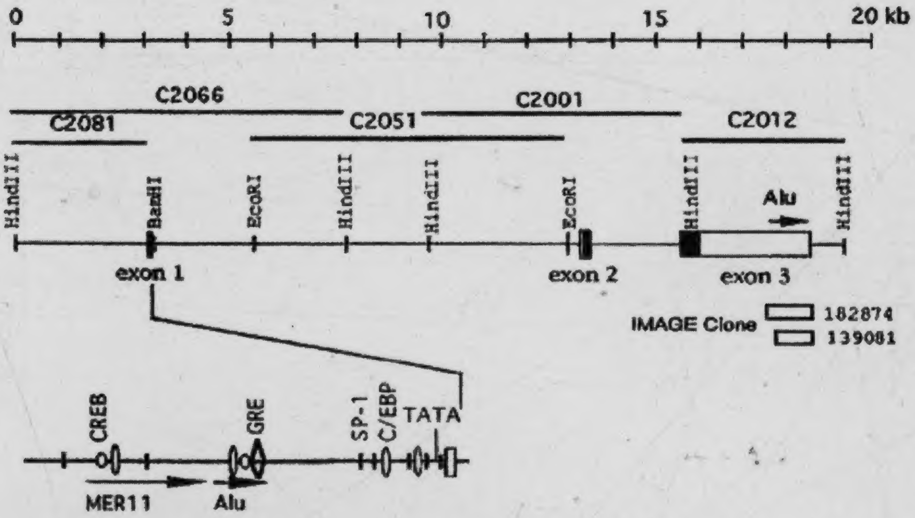
0
5
10
15
20 kb
C2066
C2081
C2051
C2001
C2012
HindIII
BamHI
EcoRI
HindIII
HindIII
EcoRI
HindIII
Alu
HindIII
exon 1
exon 2
exon 3
IMAGE Clone
182874
139081
CREB
GRE
SP-1
C/EBP
TATA
MER11
Alu

Figure 2-14 Genomic structure of human Ob gene
Restriction sites and putative regulatory domains shown.

Several regulatory elements have been mapped to the Ob gene promoter including glucocorticoid response elements (GRE), CCAAT enhancer binding protein (C/EBP) and cAMP response element binding domain (CREB, which may be a repressor in this gene). Leptin is synthesized, and secreted from white adipose tissue (primarily) (Banks et. al., 1996) and is transported in the blood to its effector organs that express its specific cell surface receptor (a product of the db gene) that are members of the cytokine class of receptors.

2.1.4.2 Leptin Activity and Regulation

Leptin has been found to be a key endocrine peptide involved in satiety, signaling of status of adiposity, and reproduction. This literature review will focus on the first two functions with little or no reference to reproduction. When exogenous leptin is delivered intravenously to ob/ob mice it was shown that the phenotypic obesity in these mice was decreased (Pelleymounter et. al., 1995:

Halaas et. al., 1995). These discoveries resulted in a premature conclusion that research might lead to a wonder drug that would render human obesity a remnant of the past. This failed to materialize at least partly because in humans most obese individuals appear to have elevated serum leptin concentrations, and appear to be leptin-resistant (Considine et. al., 1996). The secretion of leptin fluctuates diurnally with a peak occurring during the night, and its been established that plasma leptin concentrations also vary in accordance with gender and systemic adipose mass. In general females tend to have higher levels of plasma leptin whether they are of normal body weight or obese (Considine et. al., 1996). This is probably related to the effect of steroid hormone as indicated by the finding that testosterone reduces leptin mRNA levels and estrogen increases it (Wabitsch et. al., 1997: Elbers et. al., 1997). White adipose, the primary secretory tissue for leptin is well innervated sympathetically and it has been elucidated that sympathetic activation of WAT reduces Ob gene transcript (Trayhurn et. al., 1996). In support of this relationship it had been reported that cold-exposure (known to increase sympathetic activity in adipose tissue) rapidly reduces leptin and this reverses quickly upon rewarming, indicating that it is not due to reduced adiposity (Trayhurn et. al., 1995). As previously mentioned, C/EBP binding motifs are present in the promoter region of the OB gene and are known to induce gene transcription. A study by Hollenberg et. al. (1997) found that there was an antagonism of the C/EBP-mediated enhancement by PPARγ ligands (TZD's). It appears that PPARs inhibit the transcription of the leptin gene. In contrast to these studies, leptin was found to be increased by insulin (Saladin et. al., 1995), glucocorticoids (De Vos et. al., 1998), TNF-α (Grunfeld et. al., 1996), obesity and nutrient intake (Considine et. al., 1996; Saladin et. al., 1995). The next diagram borrowed from a review by Rayner and Trayhurn (2001) summarizes most of these regulators of leptin gene expression.

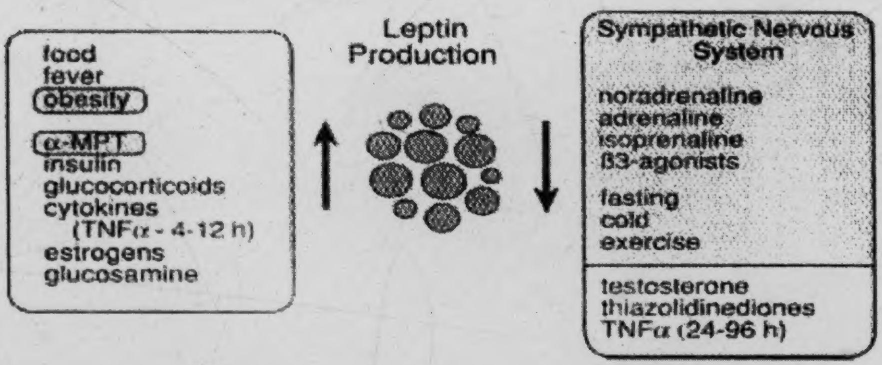
food
fever
obesity
α-MPT
insulin
glucocorticoids
cytokines
(TNFα - 4-12 h)
estrogens
glucosamine
Leptin
Production
Sympathetic Nervous
System
noradrenaline
adrenaline
isoprenaline
β3-agonists
fasting
cold
exercise
testosterone
thiazolidinediones
TNFα (24-96 h)

Figure 2-15 Factors influencing leptin production in WAT
(α-Methyl-p-transferase (α-MPT), tumour necrosis factor-α (TNF-α)

The exogenous administration of leptin to animals results in a dose-dependent reduction in food intake, body weight, and fat depots with an increase in energy metabolism (Campfield et. al., 1995; Pelleymounter et. al., 1995; Chen et. al.; 1996; Levin et. al., 1996). These studies emphasize the importance of leptin in the systemic regulation of energy balance. Leptin has central effects moderated through the hypothalamus, and peripheral effects at various tissues that express the leptin receptor. The central activity of leptin is known to involve its ability to inhibit neuropeptide Y (NPY) synthesis in the hypothalamic arcuate nucleus (Steven et. al., 1995; Schwartz et. al., 1996). This is not the complete story though because animals that are NPY-deficient still become appetite-suppressed upon leptin treatment (Erickson et. al., 1996). In the next figure the current model of central leptin and NPY interaction is outlined (Kokot and Ficek, 1999).

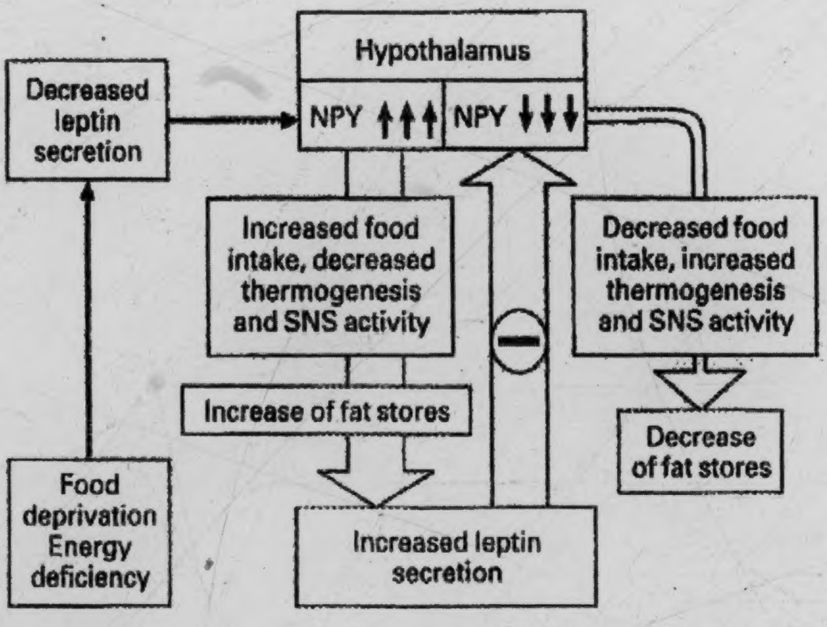
Hypothalamus
Decreased leptin secretion
NPY ↑↑↑
NPY ↓↓↓
Increased food intake, decreased thermogenesis and SNS activity
Decreased food intake, increased thermogenesis and SNS activity
Increase of fat stores
Decrease of fat stores
Food deprivation Energy deficiency
Increased leptin secretion
−

Figure 2-16 Leptin-NPY feedback under conditions of food restriction and food abundance.
↑↑↑ = increase, ↓↓↓ = decrease of NPY synthesis, – = suppressing effect of NPY synthesis and secretion.

Further evaluation has determined that many other CNS peptides are influenced by leptin. Leptin also inhibits agouti-related peptide (AGRP) another orectic CNS protein. The ability of leptin to suppress appetite is accentuated by its ability to increase expression of Cocaine-Amphetamine related transcript (CART, an anorectic) and proopiomelanocortin (POMC). Enzymatic cleavage of POMC results in several active peptides including α-MSH (alpha-melanocyte stimulating hormone; an appetite suppressor). A key role for α-MSH is the inhibition of melanin-concentrating hormone (MCH), which is an appetite-inducing peptide. Therefore, leptin suppresses appetite by reducing central orectic signals (NPY, AGRP, MCH) and simultaneously increasing anorectic signals within the hypothalamus. The highest expression of the leptin receptor is concentrated in the arcuate nucleii and the paraventricular nucleii of the hypothalamus, but peripheral tissues have also been found to express the leptin receptor indicating some peripheral function for this adipostatic hormone. Prior to reviewing the peripheral role of leptin, included is a figure that effectively reviews the centrally-mediated effects of starvation, in terms of leptins role in initiating the systemic response (Ahima and Flier, 2000).

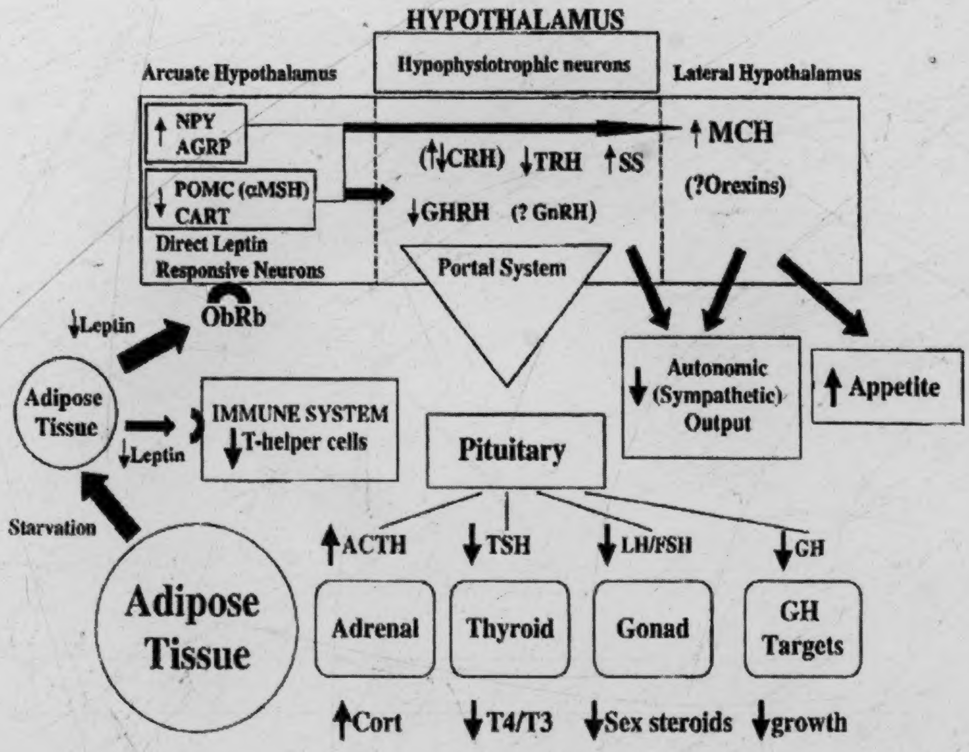
HYPOTHALAMUS
Arcuate Hypothalamus
Hypophysiotrophic neurons
Lateral Hypothalamus
↑ NPY AGRP
↓ POMC (αMSH) CART
Direct Leptin Responsive Neurons
↑ MCH
(↑↓CRH) ↓TRH ↑SS
(?Orexins)
↓GHRH (? GnRH)
Portal System
↓Leptin
ObRb
Adipose Tissue
IMMUNE SYSTEM ↓T-helper cells
↓Leptin
↓ Autonomic (Sympathetic) Output
↑ Appetite
Pituitary
Starvation
↑ACTH
↓TSH
↓LH/FSH
↓GH
Adipose Tissue
Adrenal
Thyroid
Gonad
GH Targets
↑Cort
↓T4/T3
↓Sex steroids
↓growth

Figure 2-17 Diagram of Hypothalamic signaling under starvation and satiety conditions

This figure illustrates the coordinated effects of hypothalamic signaling under conditions of starvation and leptin influence, to reduce metabolic rate (↓T4/T3 & ↓SNS), reduce growth (↓GH) and reproduction (↓LH/FSH), while stimulating the drive to eat.

Broad spectrum screening of mouse tissue has revealed a near ubiquitous expression profile for the leptin receptor, though there are several variants of this, which will be discussed later. The reputed active long form leptin receptor (OB-Rb), was found in the lung, heart, liver, spleen, various WAT depots, skin, bone, brain cortex, pituitary, adrenals, stomach, small intestine, colon, kidneys, bladder, testis and hypothalamus (Lollman et. al., 1997). Not only is the leptin receptor expressed in peripheral tissues, leptin itself is produced by tissues other than adipose. Leptin and leptin receptor are found in the stomach (Bado et. al., 1998; Sobhani et. al., 2000; Mix et. al., 2000), skeletal muscle (Wang et. al., 1998), pancreatic islet cell (Emilsson et. al., 2001), fibroblasts (Glasow et. al., 2001) and placenta (Reitman et. al., 2001). The expression of both leptin and its receptor by tissues is suggestive of it having some autocrine regulatory function related to cellular metabolism. There is good evidence to support the observation that leptin not only suppresses appetite but further increases metabolic rate to maintain homeostatic energy balance in the face of ample nutrients. Leptin increases fatty acid oxidation in liver and muscle (Muoio et. al., 1997) by a pathway involving AMP-activated protein kinase (Minokoshi et. al., 2002). Additionally, leptin increases expression of UCP-1 and UCP-3 in brown fat and thus increases thermogenesis (Scarpace et. al., 1997; Scarpace et. al. 1998), increases UCP-2 in WAT (Zhou et. al., 1997), and increases UCP-2 and UCP-3 in skeletal muscle (Cusin et. al.. 1998), when administered chronically. This interplay between leptin and uncoupling proteins suggests that they are somewhat coordinated in their influence on systemic energy balance and play key roles in dictating whether a cell is directed towards a primarily catabolic state. Another interesting observation is that leptin-resistant mice express much less uncoupling protein in their BAT, WAT and skeletal muscle (Masaki et. al., 2001), suggesting that these obese mice may be further disposed to weight gain by their inability to uncouple their mitochondria perhaps rendering them more efficient.

In contrast to many of the candidate genes discussed above there has actually been some research on domestic livestock involving leptin and its receptor(s). One study, not surprisingly found that porcine backfat thickness is positively associated with leptin mRNA levels in this subcutaneous adipose (Robert et. al., 1998), supporting the observed increase in porcine serum leptin in obese animals as compared to more lean animals (Ramsay et. al., 1998). A further study on lactating ewes in negative energy balance reports that the serum leptin was substantially reduced while the hypothalamic NPY expression was elevated (Sorensen et. al., 2002). This last study establishes that hypoleptinemia in lactating ewes may activate the appetite induction pathway mediated by hypothalamic NPY, promoting hyperphagia, suggesting that it functions in the same manner as observed in rats and humans. Houseknecht et. al. (2000), found that in bovine subcutaneous adipose tissue leptin mRNA was elevated in growth hormone-treated cattle as compared to control steers. More research is required before an effective hypothesis for this association between bovine somatotropin and leptin is established although it is known that somatotropin has a generalized stimulatory effect on adipocyte lipolytic activity (Houseknecht et. al., 1998). One other study involving cattle has reported that plasma leptin is not influenced by breed (fat Charolais vs. fat Holstein), was overall decreased by underfeeding (0.6X) and was elevated 4hours postprandially in under fed cattle (0.6X) but decreased postprandially in well fed cattle (1.3X) (Delavaud et. al., 2002).

Although, these results appear to correspond to leptin research in other species, other than the suppressed post-prandial leptin in the well-fed animals; suspicion exists with regards to these findings because their analyses relied on the use of a "Linco multi-species RIA kit", that has failed to provide linearity for all other bovine researchers.

This brief review of leptin serves to reinforce its selection as a candidate in livestock that may be important in the whole animal metabolism. In its simplest form, if cattle were to express less leptin they may eat more, develop higher adiposity and become slightly more efficient through their reduced lipid turnover. Of critical importance to the action of leptin is the expression of its receptor, and as such this was another of the candidate genes identified for the current study.

2.1.5 Leptin Receptor

In 1995, the receptor for leptin was discovered and at the same time shown to be responsible for the phenotype of obese db/db mice (Tartaglia et. al., 1995). The db/db mice express a truncated version of the receptor that retains both its ligand binding and its transmembrane domains but lacks the intracellular signaling domain and thus does not appear to initiate any signalling cascade (Chen et. al., 1996). As mentioned previously, the leptin receptor protein is found directly on the NPY-containing neurons in the arcuate nucleus of the hypothalamus (Baskin et. al., 1999) and on hypothalamic POMC-containing neurons (Cheung et. al., 1997). The next figure borrowed from Schwartz et. al. (1997) illustrates how the db/db mice have a deficient pathway for leptin signaling in the hypothalamus.

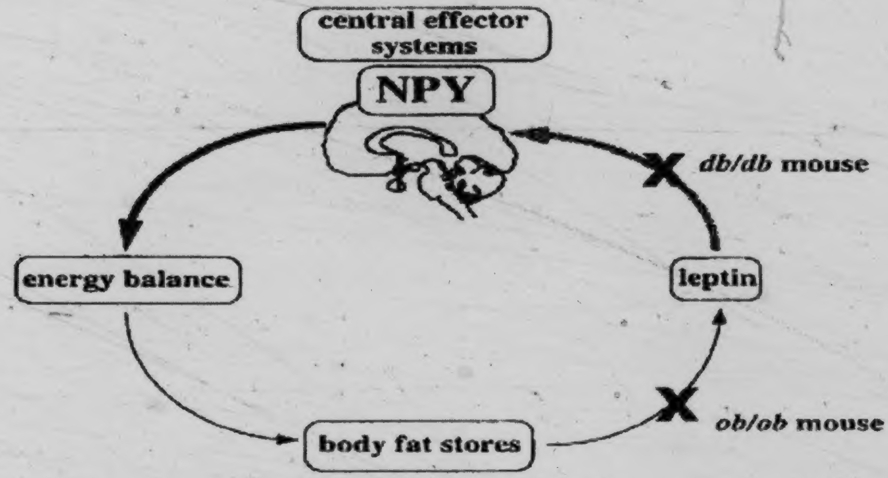

Figure 2-18 Model of negative feedback regulation of adiposity, with labelled positions of genetic deficiencies of two obese mouse strains.

The leptin receptor is a member of the class I cytokine family of receptors that are activators of the Janus kinase (JAK)/ Signal transducer and Activator of transcription (STAT) signal transduction pathway. A number of splice variants of the leptin receptor have been described to date. This review will focus on three of these variants: OB-Rb, OB-Ra, and OB-Re, because these splice variants are the only ones with putative roles that involve leptin function.

2.1.5.1 Leptin Receptor Characteristics (OB-Rb, OB-Ra, and OB-Re)

The three isoforms of leptin receptor that are reviewed in this section are all generated as splice variants of the same gene. The leptin receptor gene has been mapped to bovine chromosome 3 (chromosome 1 in humans). The leptin receptor gene contains 20 exons and these exons encode a protein with an 816 aa extracellular domain, a single 23 aa transmembrane domain and a 303 aa intracellular component (in the longest OB-Rb isomer) (Heshka and Jones, 2001). Its ligand binds to two regions in the extracellular domain of each of the homodimeric functional units and induces conformational changes in the receptor (Banks et. al., 2000). The longest form of the receptor (OB-Rb) is the isoform that is expressed on the surface of hypothalamic neurons and believed to be of primary importance to the ligand binding-induced activation of the JAK/STAT signal transduction pathway. The following diagram offers a structural comparison between the three isoforms to be reviewed, it was borrowed from a review by Heshka and Jones (2001).

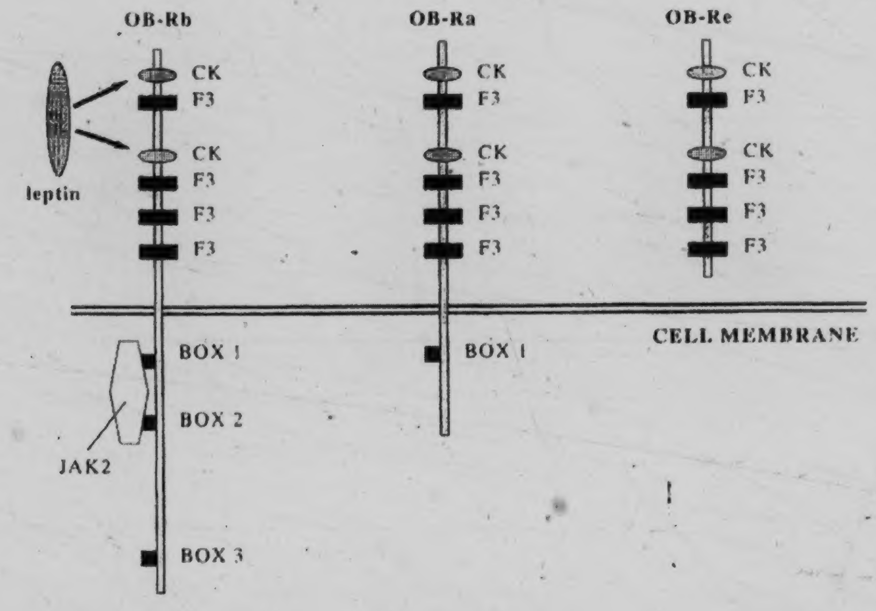

Figure 2-19 Comparison of the leptin receptor isoforms. The long form of the receptor (OB-Rb) is generally considered the functional isoform.

The OB-Ra isoform of the receptor is missing a portion of the intracellular domain essential for activation of the JAK/STAT pathway and so is not believed to be functional in this function. Alternatively, it has been shown that the Ob-Ra retains its extracellular ligand binding capacity and that this receptor isoform is hypothesized to be of critical importance to the transfer of leptin across the blood brain barrier.

2.1.5.2 Leptin Receptor Activity

As mentioned in the brief introduction to the leptin receptor, the long form (OB-Rb) is present as a homodimer on the extracellular surface and undergoes a conformational change upon ligand binding. This ligand-induced change allows for the autophosphorylation of Janus Kinase (JAK) that is bound to the intracellular domain of the OB-Rb receptor (Ghilardi and Skoda, 1997). The activated JAK can then catalyze the tyrosine phosphorylation of STAT proteins (Vaisse et. al., 1996). Phosphorylated STAT proteins can then act as transcription factors influencing the expression of genes within the activated cell. It has further been demonstrated that JAK/STAT can activate extracellular factor-regulated kinases (ERKS) that are serine/threonine kinase, these activated ERKS translocate to the cell nucleus and mediate gene expression (Banks et. al., 2000). A figure borrowed from the review by Heshka and Jones (2001) models the signal transduction pathways utilized by the activated OB-Rb receptor.

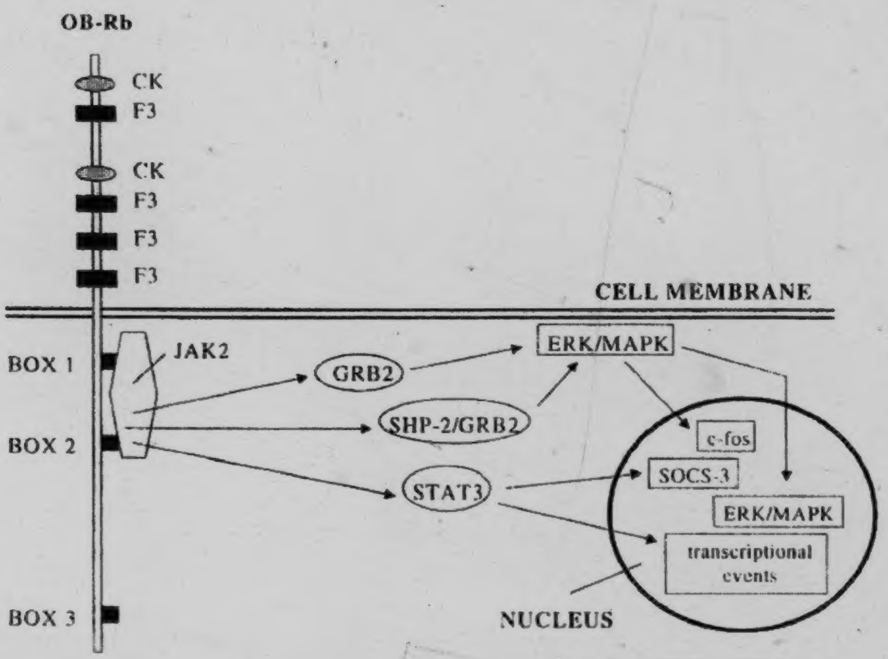
OB-Rb
CK
F3
CK
F3
F3
F3
CELL MEMBRANE
BOX 1
BOX 2
BOX 3
JAK2
GRB2
SHP-2/GRB2
STAT3
ERK/MAPK
c-fos
SOCS-3
ERK/MAPK
transcriptional events
NUCLEUS

Figure 2-20 Model of signaling by OB-Rb. Coserved BOX 1 and Box 2 motifs interact with JAK2 and upon ligand stimulation phosphorylate intracellular tyrosine residues. GRB2 is activated by phosphorylation resulting in transcription of ERK/MAPK and cfos accumulation. Phosphorylated STAT3 mediates SOCS-3.

The previous figure only shows activation of STAT3 but it has been shown that leptin receptor interacts with STAT1, STAT3 and STAT5 (Bjorbaek et. al., 1997). This interaction was shown to be involved in the fatty phenotype of the fa/fa rats, where an amino acid substitution (Philips et. al., 1996) impairs the mutant leptin receptor's ability to activate STAT5 (Takaya et. al., 1996). In order to determine the characteristics of the leptin receptor and STAT 3 interaction, mutant mice (lepr s/s mutants) were created that had an amino acid substitution in the leptin receptor that specifically disrupts its interaction with STAT3 (Bates et al, 2003). Studies using these mutants suggest that the leptin receptor-STAT3 interaction is responsible for leptin's effects on the melanocortin system and body energy homeostasis, but not regulation of NPY, growth, fertility and glucose homeostasis (Bates et. al., 2003). Unlike the infertile, short and diabetic db/db mutants these mutants were fertile, long and less hyperglycaemic while sharing hyperphagic and obese phenotypes similar to the db/db mice (Bates et. al., 2003).

Using in situ hybridization and/or RT-PCR the long form leptin receptor has been found localized in the arcuate nucleus and paraventricular nucleus of the hypothalamus (Mercer et. al., 1996; Huang et. al., 1996; Fei et. al., 1996), brown and white adipose (Kielar et. al., 1998; Kutoh et. al., 1998) liver and muscle (Muoio et. al., 1997). Furthermore, mass screening of mouse tissue using RT-PCR and RNase protection assay has shown the active long form leptin receptor (OB-Rb), to be present in the lung, heart, liver, spleen, various WAT depots, skin, bone, brain cortex, pituitary, adrenals, stomach , small intestine, colon, kidneys, bladder, testis and hypothalamus (Lollmann et. al., 1997). Therefore, it is likely that leptin exerts some influence on peripheral tissues outside the central mediation of satiety, and this is likely to involve its specific receptor and its relative expression on the cell surface.

The significance of the leptin receptor to the leptin-mediated regulation of nutrient intake is emphasized by the db/db obese mice and the fa/fa obese rats but also by other studies.

For instance a spontaneous mutation resulted in an obese and hypertensive rat, that was determined to have a null leptin receptor mutation (Wildman et. al., 2000). Furthermore, a single nucleotide polymorphism in the leptin receptor of postmenopausal caucasian women has been well correlated with BMI, fat mass and leptin levels indicating that it may represent a scenario of reduced insulin sensitivity (Quinton et. al., 2001).

Scarpace et. al. (2001) found that infusion of leptin into young vs. aged obese rats resulted in vast differences in the measured STAT3 activation in the hypothalamus. This led to the conclusion that an impaired leptin signal transduction in the aged rats, was indicative of leptin resistance and resulted in the adult onset obesity, and hyperphagia in these animals (Scarpace et. al., 2001). In stark contrast an observed increase in hypothalamic expression of the OB-Rb-receptor in NPY knockout mice is associated with leptin hypersensitivity and fasting (Baskin et. al., 1998). These publications support the concept of an OB-Rb mediated response in the arcuate nucleus mediating the anorectic function of leptin and that abnormalities in the receptor or its expression result in abnormal appetite related traits.

As alluded to earlier, the shortened isoform of the leptin receptor Ob-Ra is abundantly expressed in the leptomeninges and the choroid plexus of the brain which is considered important to the transfer of leptin across the blood brain barrier (Banks et. al., 1996; Golden et. al., 1997). It was further suggested that this transport is likely saturable and may be linked to the putative leptin resistance in obese humans (Banks et. al., 1996). There is some recent supportive research for this theory, such as the finding that injection of a bolus of radiolabelled leptin resulted in a much reduced presence of labelled leptin in the CNS in obese rats than in normal rats (Burguera et. al., 2000). Additionally, in vitro studies have recently shown that OB-Ra can transport leptin across a transcellular surface of a monolayer of Madin-Darby Canine Kidney cells (MDCK) (Hileman et. al., 2000).

Finally the OB-Re truncated isoform of the leptin receptor contains no transmembrane domain and thus is a soluble (non-membrane bound) receptor. It is believed to act as a plasma-binding protein for leptin, and perhaps assist in the delivery of leptin to the blood-brain barrier. Not much research involving this isoform has been performed to date, though it may have similar regulatory aspects regarding leptin function through increasing half-life, changing activity in bound vs. free form, and or quenching serum leptin concentration in various physiological states (i.e. pregnancy). There is a strain of obese rodents with a genetic mutation that results in their lacking all but the soluble receptor and these mice have normal elevated physiological levels of leptin but were resistant to its actions (Lahlou et. al., 2000). This indicates that the obese mice elevated their serum leptin as would be expected for their increased adiposity but that they were unresponsive to it action. This isoform may also be extremely important in the development of leptin resistance in some individuals and may also assist in explaining why some individuals are more leptin-sensitive than other individuals.

The regulation of leptin receptor gene expression, regardless of which isoform, will likely prove to be an important factor in the orchestration of the normal balance between orectic and anorectic signals. It is postulated that NPY itself suppresses leptin receptor expression and this is supported in principal by the elevated OB-Rb expression in NPY knockout mice (Baskin et. al., 1998). It is also suggested that the activation of leptin receptor itself acts as an autoregulator of its gene expression as induction of the "Suppressor of Cytokine Signaling (SOCS)" may suppress leptin receptor gene transcription (Bjorbaek et. al., 1998).

As has been mentioned, a great deal of the leptin-mediated influence on appetite and nutrient intake are coordinated in the hypothalamus and are correlated to the orectic signaling especially that mediated by neuropeptide Y (NPY). It is not surprising that we therefore included NPY was included as one of the candidate genes and it will be briefly reviewed in the following section.

2.1.6 Neuropeptide Y (NPY)

The association of neuropeptide Y with appetite has existed longer than knowledge about leptin. In 1984, research showed that central administration of NPY in rats could induce excessive eating (Stanley et. al., 1985). Furthermore, in line with the up-regulation of NPY during acute fasting it has also been established that NPY mediates the initiation of reduced energy expenditure in an attempt to conserve energy. This was shown by Billington et. al. (1991) who observed a decrease in brown fat thermogenesis and UCP-1 expression in centrally-infused NPY mice. It appears that NPY is the flip-side of the leptin coin, in terms of their individual effect on both nutrient intake and energy expenditure.

2.1.6.1 Characteristics of NPY

NPY is a peptide consisting of 36 amino acid residues that arises through several post-translational processing steps of the original NPY gene product "pre-pro-NPY" that is 97 amino acid residues in length through a 69 amino acid intermediate; "pro-NPY". This product is translated from a 551 bp mRNA transcribed from the four exons of the NPY gene. The figure included on the next page is from a review by Cerda-Reverter and Larhammar (2000) outlines the relationship between the final NPY and the gene and its original translational product.

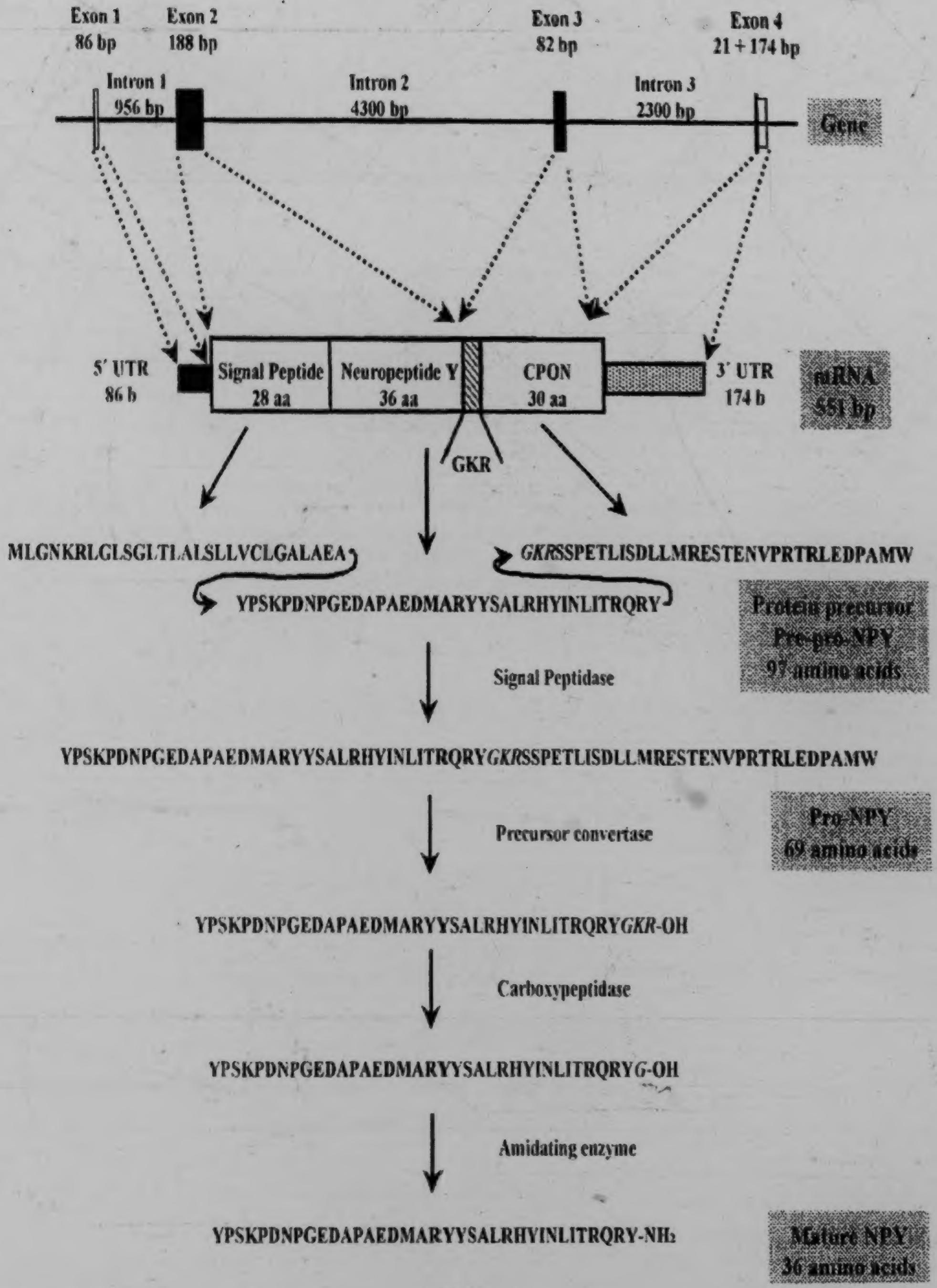
Exon 1
86 bp
Exon 2
188 bp
Exon 3
82 bp
Exon 4
21 + 174 bp
Intron 1
956 bp
Intron 2
4300 bp
Intron 3
2300 bp
Gene
5' UTR
86 b
Signal Peptide
28 aa
Neuropeptide Y
36 aa
CPON
30 aa
3' UTR
174 b
mRNA
551 bp
GKR
MLGNKRLGLSGLTLALSLLVCLGALAEA
GKRSSPETLISDLLMRESTENVPRTRLEDPAMW
YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY
Protein precursor
Pre-pro-NPY
97 amino acids
Signal Peptidase
YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRYGKRSSPETLISDLLMRESTENVPRTRLEDPAMW
Pro-NPY
69 amino acids
Precursor convertase
YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRYGKR-OH
Carboxypeptidase
YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRYG-OH
Amidating enzyme
YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH2
Mature NPY
36 amino acids

Figure 2-21 Schematic of human NPY gene, mRNA and protein precursor.

2.1.6.2 NPY Activity and Regulation

Circulating NPY is released from the sympatho-adrenomedullary nervous system (Gray and Morley 1986), but this is impermeable to the blood brain barrier. Therefore, the central orectic pathways are reliant on the production and release of NPY from the arcuate nucleus of the hypothalamus. The NPY receptors subtypes 1 and 5 are located in the paraventricular nucleii of the hypothalamus and are thus responsive to the release of NPY from the axonal projection of neurons originating in the arcuate nucleus (Schwartz et. al., 1995, Woods et. al., 1998). The paraventricular nucleus is a major site of integration of afferent inputs related to energy homeostasis and coordinated outflow of SNS fibres to various effector organs. The release of NPY into the PVN acts in a concerted fashion to suppress sympathetic outflow while increasing parasympathetic outflow. This explains NPY-mediated inhibition of sympathetic outflow to brown adipose with simultaneous induction of lipogenic enzymes in white adipose (Woods et. al., 1998). Since other orectic pathways are present NPY knockout mice are not anorexic but are very leptin sensitive and, due to another NPY-mediated function not reviewed here, they are extremely prone to seizures (Erickson et. al., 1996). The next figure illustrates the hypothalamic NPY pathway involved in feeding (Schwartz et. al., 1997).

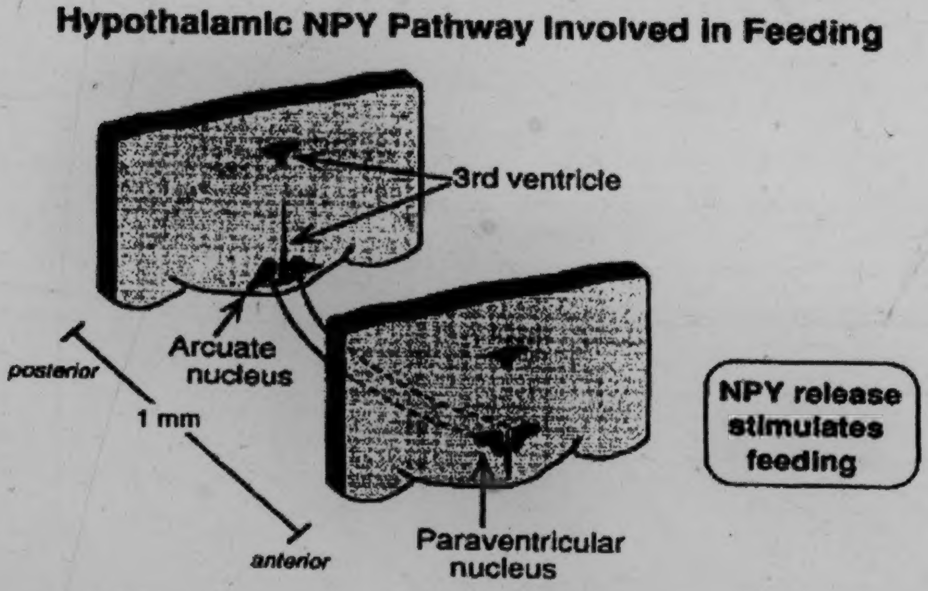
Hypothalamic NPY Pathway Involved in Feeding
3rd ventricle
Arcuate nucleus
posterior
1 mm
anterior
Paraventricular nucleus
NPY release stimulates feeding

Figure 2-22 Model of Hypothalamic NPY pathway in control of energy balance

Surprisingly, very little to date is known about the promoter region of the NPY gene. Though it is known that activation of leptin receptors in the hypothalamus result in an inhibition of NPY mRNA, the exact mechanism for this response remains unknown. Though it is purported that the negative regulatory element responsible for this exists in a portion of the NPY promoter located between –798 and –498 bp relative to the transcription start site, this hasn't been directly identified and mapped (Minth-Worby, 1994). It is proposed that the leptin receptor activation of SOC3 as well as c-fos, are important in the NPY regulation (Elias et. al., 1999) but again this has not been scientifically substantiated.

The regulation of NPY is also affected by ambient temperature. It was shown that acute cold exposure (2.5-18 hours) reduced NPY, allowing the sympathetic activation of brown fat, increased UCP and thus elevated thermogenesis (Bing et. al., 1996). In contrast, longer term cold exposure was found to elevate NPY and is hypothesized to support the increase in dietary intake associated with cold (Kotz et. al., 1998).

Prolonged central administration of exogenous NPY has been shown to promote adipose accumulation and ultimately result in obesity (Stanley et. al., 1986, Zarjevski et. al., 1993). In the study by Zarjevski et. al. (1994) they also noted that in spite of a vast increase in insulin-stimulated glucose uptake in adipose tissue the central infusion of NPY resulted in reduced glucose uptake in skeletal muscle. This phenomenon could not be explained by differences in Glut-4 (Insulin-sensitive glucose transporter) expression and may relate to varied innervation arising from the paraventricular hypothalamus to these two effector organs. This group hypothesized that the increase in muscle triglycerides in the NPY-infused mice may inhibit their uptake of glucose, a scenario that may be in effect in NIDDM subject as well (Zarjevski et. al., 1994). One further study found that NPY expression in response to high fat vs. high carbohydrate diets consistently showed reduced hypothalamic NPY in the high fat diet animals as compared to the high carbohydrate diet (Giraudo et. al., 1994). The post-prandial increase in both insulin and leptin are negatively correlated with the hypothalamic NPY expression and this is illustrated in the following figure by Schwartz et. al. (1997).

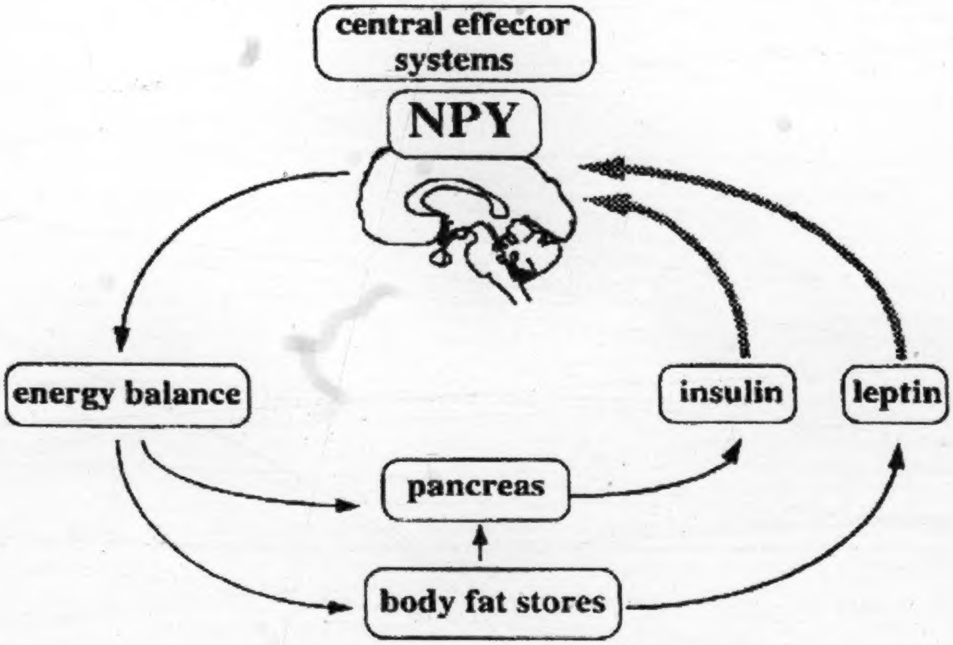

Figure 2-23 Model of integration of two, long-term adiposity signals in the regulation of systemic adiposity.

One final physiological function of NPY that is directly related to animal metabolism is related to the discovery that NPY-containing neurons synapse with Thyrotropin-releasing hormone (TRH) neurons present in the paraventricular nucleus (PVN) of the hypothalamus (Legradi and Lechan, 1998). This study suggested that NPY may be involved in suppressing the release of TRH from neurons that it forms synapses within the PVN. Since TRH is the primary regulatory factor for anterior pituitary release of TSH, this may directly influence the thyroid activity and hence the metabolism of the animal. This appears to be a plausible pathway through which an animal faced with starvation may modify its metabolism so as to conserve valuable energy reserves.

Given that the activity of NPY is mediated by the expression of its receptors we chose to include the NPY receptor was included as a candidate gene in the current study. Although NPY receptor expression was only being measured in peripheral tissues, it was believed that this may still be important to compare with measurements of leptin receptor in the same tissues. It was speculated that the expression of the receptor genes (leptin and NPY) within tissues represent the set point of responsiveness of an animal to the influences of these endocrine peptide ligands. More specifically, an attempt was made to characterize the expression of these genes and to determine if the level of gene expression could be correlated with metabolic status of cattle and hence be related to indices of metabolic rate and/or energy conversion.

2.1.7 Neuropeptide Y Receptor(s)

There are currently six different NPY receptors identified, typically referred to as Y1, Y2, Y3, Y4, Y5, and Y6. Only receptor types Y1 and Y5 have been implicated in the feeding and obesity-related associations with NPY (Malmstrom, 2001, Marsh et. al. 1998). Interestingly, these two receptors have overlapping gene structure and are products of bi-directional transcription of the two strands (Herzog et. al., 1997). Given this unique gene relationship it is believed that they undergo coordinated transcriptional regulation. These receptors belong to the G protein-coupled receptor family.

2.1.7.1 Characteristic of NPY Receptors

The gene cluster responsible for the transcription of NPY receptors Y1 and Y5 has been mapped to the human chromosome 4 but not yet mapped to the bovine chromosomes. These receptors are GI –coupled and thus are members of this class of receptors (that includes the rhodopsin receptor), they have six transmembrane domains and induce ligand-mediated increase in cGMP intracellularly. It is typical of G_I –coupled receptors to exert an inhibitory effect on the cells that express the receptor and this may be indicative of the primary effect of NPY in the PVN as a suppressor of sympathetic outflow. The following figure borrowed from Herzog et. al. (1997) illustrates the gene cluster for human NPY Y1 and Y5. Both of these receptors have been localized in the hypothalamus and so are likely involved in the central NPY- mediated responses. Another study has found that the mRNA encoding the Y1, Y2, Y4, and Y5 receptors are present in the rat intestine and colon indicating a peripheral NPY activity.

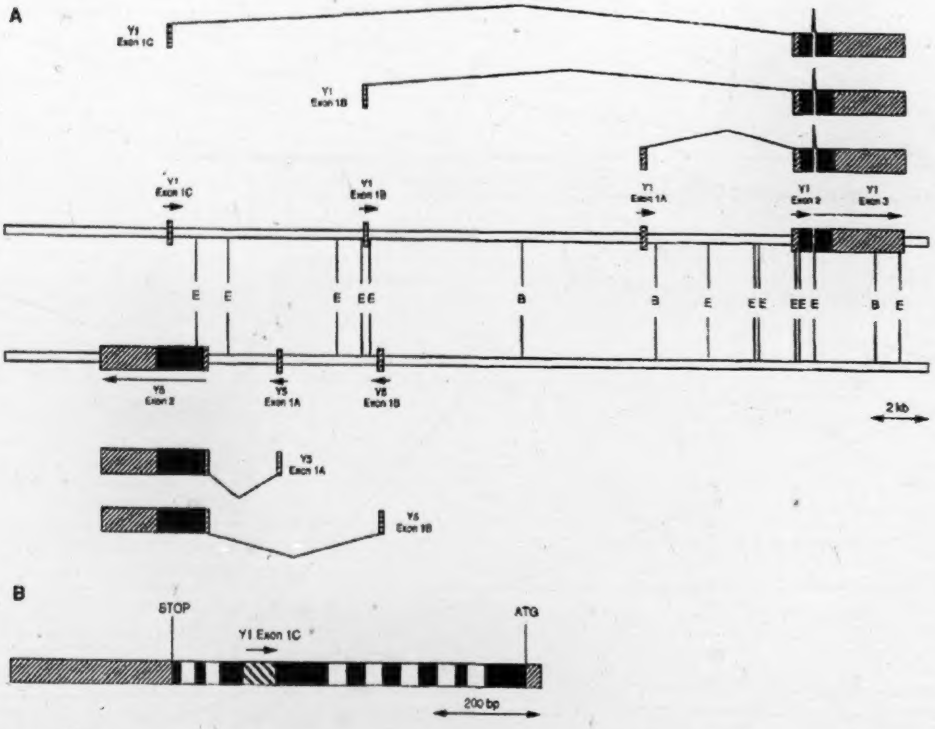
A
Y1 Exon 1C
Y1 Exon 1B
Y1 Exon 1C
Y1 Exon 1B
Y1 Exon 1A
Y1 Exon 2
Y1 Exon 3
E E E EE B B E EE EE E B E
Y5 Exon 2
Y5 Exon 1A
Y5 Exon 1B
2 kb
Y5 Exon 1A
Y5 Exon 1B
B
STOP
ATG
Y1 Exon 1C
200 bp

Figure 2-24 Schematic of the human Y1 and Y5 gene cluster showing exons and alternative splicing.

Very limited information is known about the regulation of these receptors, but it is known that there is not a compensatory up-regulation of these receptors in NPY knockout mice and that in Y5 receptor knockout mice the Y1 receptor is not up-regulated in a compensatory fashion (Marsh et. al., 1998). In a recent experiment, it was found that the Y1 receptor was decreased by starvation in the PVN but not in the arcuate nucleus, but treatment of drinking water with 10% (w/v) glucose resulted in elevated Y1 receptor expression in both the PVN and the arcuate nucleus (Zammaretti et. al., 2001). Additionally, the Y1 receptor subtype mRNA was up-regulated in association with transient hyperphagia and body weight gain which was suggested as evidence of the hypothalamic-mediated development of leptin resistance (Kalra et. al., 1998). These experiments emphasized the influence of nutrient availability on NPY receptor expression in the central hypothalamic center but a great deal more research is required before a sound understanding of how the coordinated activity of leptin, NPY and other orectic and anorectic signals are integrated.

2.2 Summary of Review

The intent of this review was to summarize some of the literature that relates to the selection of the candidate genes in this study. Although, the evaluation of the expression of these genes in beef cattle will supplement the scientific literature that currently focuses on other species, it was also performed such that it may assist in the eventual discovery of a useful bovine marker for energetic efficiency. From a production perspective, the goal is to obtain the maximum retained energy (RE) for the given amount of nutrient provision. As mentioned, the two choices available to affect RE are to reduce heat production, or increase the total energy ingested. The uncoupling proteins were selected as candidates because they were potentially related to the degree of mitochondrial inefficiency, but it should also be recollected that their expression is also influenced by the nutrient and metabolic status of the animals. The physiological function of the UCPs may be important in non-shivering thermogenesis, prevention of excessive ROS production, as well as influencing the individual cell metabolism be they in a catabolic or anabolic state.

Additionally, we sought to determine the expression patterns of leptin and neuropeptide Y, and their respective receptors, were examined on the basis of their relationship to the central control of nutrient intake. It is apparent that some chronic ailments in which we observe varied nutrient partitioning, such as NIDDM, are known to affect (or perhaps result in part from) changes in the expression profiles of these orectic and anorectic peptides.

Finally, a decision was made to evaluate the expression of these genes in several tissues in which it was expected that they might exert significant influence on the animal's metabolic processes and efficiency. The sampling of various adipose depots, mixed fibre type skeletal muscle, liver, cardiac muscle and regions of the digestive tract, would allow a broad spectrum evaluation of the expression of these genes in metabolically important tissues. The subsequent analyses of the candidate gene mRNA expression using RT-PCR was expected to be of sufficient sensitivity to allow evaluation of differences between, breeds, and treatments such as; diet composition, feeding level and varied ambient temperature.

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CHAPTER 3

Uncoupling Proteins 2 and 3 mRNA Expression in Bovine Tissues and Their Relationship to Breed, Feeding Level and Measurements of Energetic Efficiency

3.1 Introduction

There are useful means of indirectly ascertaining measures of efficiency of feed conversion in cattle, be it through digestibility determination, heat production or growth measurements, or assessment and comparison of all of these parameters. What becomes evident to both the producer and the researcher is that variability in animal feed utilization is affected by environmental parameters as well as individual animal differences, and that certain conditions and some individual animal's physiological status can promote or impede profitability. Differences between individual animals may be consistently apparent or may become apparent only upon animal adaptation to alterations in environment, diet or physiological state. That is, the adaptive strategy, and in fact the degree of adaptation of one animal compared to another may differ in response to the same stressor and this may ultimately affect the feed utilization when compared in the two animals. These adaptations emphasize the homeorrhetic status of animals, be they responding with post-prandial metabolic adaptation, compensatory growth or even physiological adaptation to immune challenge. The pertinent aspect of genetic variation in livestock becomes physiologically important when either there are transcript variations, such as polymorphisms or when there are significant expression differences, and these variations influence animal health, growth and metabolism.

Given the essential nature of many gene products for animal health and survival it is not surprising that the frequency of genetic variation that results in transcript polymorphism is relatively low and therefore it becomes necessary to evaluate tissue-specific gene expression variations as the primary source of inter-animal variation. In the context of physiology, it is understood that the various tissues within an animal emphasize the importance of varied gene expression profiles. The inherent differences between cells of different organs within the same animal are not a result of genetic differences, since all somatic cells within an animal have the same genotype, but rather an obvious and functionally important variation in the pattern of genes expressed and the associated function of the gene transcripts present. Just as varied tissues within an animal showed differing gene expression profiles which relate to their predominant biochemical and biological functions it is also true that the same tissues from different animals may show comparatively different gene expression profiles that relate to potential differences between their own specific physiological function for that tissue. The possibility that differences in tissue gene expression between animals also contributes to variations in differences in measured feed efficiency becomes plausible, especially if the tissues examined represent organs that are "highly metabolically active" such as liver, muscle, and adipose to name a few. Since uncoupling protein 1 expression has been hypothesized to affect mitochondrial proton gradients and heat dissipation, it may influence measures of metabolic feed conversion in livestock. UCP-2 is a more recently discovered gene that is expressed in many tissues in mice and humans, but has not yet been elaborately studied in cattle (Fleury et. al., 1997; Gimeno et. al., 1997). UCP-3 has also been studied recently in rodents and humans and is expressed in both skeletal muscle and brown adipose tissue but not other organs (Boss et. al., 1997, Vidal Puig et. al., 1997). UCP-3 has not yet been well studied and characterized in cattle. UCP-1, UCP-2 and UCP-3 are linked to proton-conductance pathways in mitochondria of cells and can divert energy from productive processes to heat production. It is becoming apparent that these signaling mechanisms link the central nervous system with information feeding back from peripheral tissues. These genes and their protein products could each have an influence on maintenance requirements and energy expenditure / efficiency in animals (Enerback et. al., 1997, Fleury et.al., 1997). They may be of particular importance in ruminants since, in these species, the energetic efficiency of growth is lower than in monogastric animals. Therefore a decision was made to test several hypotheses regarding gene expression and energy metabolism. It was hypothesized that these genes would be expressed in variable amounts in bovine tissues and that the mRNA expression profile might reflect the individuality of each steers adaptive state. Further it was hypothesized that there might be differences in tissue specific expression of these genes that relate to within breed, between breed, and adaptive variability in response to different feeding levels. Finally the hypothesis was examined that the tissue specific mRNA expression of the candidate genes might be influential and thus correlated with measures of metabolic efficiency and heat production.

3.2 Material and Methods

3.2.1 Animals and Treatments

Thirty-six cattle, 12 from each of three breed types (15-17 months); Charolais {503.7kg +/- 40.3kg}, Angus {459.3kg +/- 37.6kg}, and Brahman/Angus (Brangus) cross {405.9kg +/- 24.9kg} were utilized in this experiment. These cattle were maintained at the University of Alberta Environmental and Metabolic Research Unit and experiments were conducted using techniques approved by FAPWC and UAPWC as being in accordance with CCAC guidelines (CCAC, 2003). The selection of breeds was designed to give a higher probability of measured differences, due to differences in size and genetic and phenotypic attributes. However these animals were acquired from individual herds and, as such, are representative of similar animals but can not be considered as absolute references of their given breeds. Starting at 15-16 months of age, the steers were fed either restricted (1.2X maintenance) or high intake levels (2.2X maintenance) of a standard feedlot diet (approximately 90% w/w Barley conc., 10% w/w roughage, this mixed diet contained, on average, ME 14.496 MJ/kg of DM; CP 14.93 % w/w DM) for two periods of 45 days each in a crossover design. Diet ME values were estimated and prepared for each animal initially using NRC (1984) feed guidelines to establish intakes. Animals were individually housed (18 ± 2 °C room temperature) in floor pens (3 m x 3 m) with wood shavings as bedding. Feed was offered to the animals between 0800 and 0900 h daily and orts were collected and weighed. Prior to the commencement of the study the steers were fed in groups and had constant access to water throughout the adaptation period. Animals were weighed before they were placed on the treatments and at two weeks intervals. Before the start of period one, animals were gradually introduced to the concentrate diet, and were allowed a further 2 weeks of adaptation once the 2.2M groups achieved satisfactory intake. After the completion of period one, feed supply to the 1.2M animals that were being transferred to 2.2M was gradually increased while those from 2.2M in period one was gradually brought down to 1.2M. The adaptation period ranged from 14 to 16 days at each feeding level before animals were studied in calorimetry hoods. Energy expenditure measurements (respiration calorimetry) were made during the last 10 days of each period.

While in the respiration hoods the animals had access to both feed and water though their mobility was limited to standing, laying, feeding, drinking and minor postural adjustments. Biopsy samples of the biceps femoris muscle (0.5-1.0g) were taken from each of the treatment animals, within 3 days following to completion of calorimetry measurements in each period. Total RNA was isolated from each tissue for assessment of gene expression using RT- PCR and semi-quantification of the specific cDNA amplicons. After period 2, animals were kept on their respective feeding levels up until the day of slaughter. Samples of muscle, adipose tissue and selected internal organs (liver, heart, duodenum, and rumen papillae) were collected, at slaughter, for isolation of RNA to study gene expression in conjunction with the metabolism measurements (detailed protocols for biopsy and post-mortem tissue collection are included in the Appendix).

3.2.2 *Assessment of Energy Utilization*

Feed intakes were measured daily from the start of adaptation to the end of each experimental period. Fecal excretion of energy and nitrogen were determined by reference to lignin and acid-insoluble ash in feed and fecal grab samples collected over a three-day collection during each period. Urinary excretion of energy, and nitrogen were determined during three days of total urine collection. Detailed methodology for calorimetry, digestibility, urine collection and laboratory analyses is described in Appendix two.

Heat production (energy expenditure) was recorded on individual animals using respiration calorimetry while the animals were fed on different planes of nutrition (estimated 1.2X and 2.2X maintenance using NRC guideline). These data provided estimates of energy retention, maintenance requirement and efficiency of utilization of retained energy and of protein retention. At slaughter, data was obtained on carcass grade and fat.

3.2.3 *Gene Expression Studies*

3.2.3.1 RNA Extraction

Total RNA was extracted from measured quantities of frozen powdered tissue samples for each individual animal using a modified Chomczynski protocol (1994) and Gibco Trizol^{TM} phenol-chloroform phase extraction procedure. The integrity of each Total RNA sample was examined using electrophoresis on acrylamide gels and examination of the abundance and integrity of the 18S and 28 S ribosomal RNA bands. The total RNA purity and quantity were determined for each sample using absorbance measurements at wavelengths of 260nm and

280nm, and the respective ratio of these measures. Working stocks (50μl) of 1μg total RNA/μl for each sample were prepared using DEPC-treated water. These working stocks were utilized for the reverse transcription and subsequent PCR analyses.

3.2.3.2 Reverse Transcription and Polymerase Chain Reaction

Reverse transcription was performed for each sample using 1ug total RNA, oligo-dT^{15} primer and Gibco MMLV-RT enzyme, in the presence of RNase inhibitor (Gibco RNAase OUT) at 37 ° C for 60 minutes in a thermocycler (Perkin Elmer 2400 or BioRad icycler). Additional details regarding the reverse transcription protocol are included in the Appendix. Each reverse transcription reaction supplied template for a minimum of three specific multiplex PCR reactions using specific primer sets and Taq polymerase enzyme (Sigma). Sense and antisense primer sets were designed for each of the specific target genes using Genbank as a resource for specific bovine gene and mRNA sequences. Genejockey and Amplify software were utilized to design and conceptually test each of the primer sets. The inherent principal of primer design is such that if sense and antisense primer sets are created that have 100% sequence homology to a known gene's messenger RNA and, given that each primer is about 20 bases long, annealing of such primers during PCR is specific and preferential to the intended gene's mRNA and as such will result in amplification of only that message. Our optimized primer sets are able to specifically amplify and thus semi-quantify bovine mRNA representing the following genes; uncoupling protein 1(UCP1), uncoupling protein 2 (UCP2), uncoupling protein 3 (UCP3).

Further, we have utilized primers sets for constitutively expressed genes, either glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and cyclophillin as our internal control in each of our specific relative reverse transcription polymerase chain reactions (relative RT-PCR). This internal control normalizes for slight variations in reaction efficiency, RNA loading and pipetting. All PCR reactions were performed on either a Perkin Elmer 2400 thermocycler or BioRad Icycler. The products of the PCR reactions (amplicons) were electrophoresed on a 2% (w/v) agarose /TBE gel along side a molecular weight marker (Gibco 100bp DNA ladder). The gels were stained using ethidium bromide (0.1% w/v) so as to observe the PCR products under UV fluorescence. The specific amplicon bands could thus be relatively quantified generating optical density scores for the amplicon bands using BioRad's Gel Doc and Molecular Analyst software.

Expression of messenger ribonucleic acid (mRNA) for specific candidate genes was measured by relative RT-PCR analyses using primer sets designed with homology to known bovine UCP 2 and UCP-3 gene sequences. These primers were designed to include a trans-intronic region of the gene sequence so that the mRNA amplicon would definitively be smaller than an amplicon generated from genomic DNA contamination. The RT-PCR reactions were validated for linearity with regards to total RNA quantity and cycle number and the amplicons were sequenced to validate the specificity and selectivity for the desired uncoupling protein isoform. Comparisons of relative gene expression for individual animals could thus be compared to other parameters including measured indices of metabolic rate such as indirect calorimetry heat production and indices of intake and efficiency such as metabolizable energy and energy retention values. Through our cross-over design we compared gene expression profiles of these candidates under the influence of the two different feeding levels in *biceps femoris* skeletal muscle. Additionally we examined mRNA expression differences within and between three breeds of cattle to determine to what extent apparent variations in efficiency were related to individual gene expression of these selected genes in the metabolically important tissues sampled. Further, analyses of these candidate genes in the various tissues collected *postmortem* allow some comparisons to be made between the different gene expression profiles over several metabolically important tissue types. Moreover, this study represents the measurement of many of these candidate genes and their tissue expression profiles for the first time in bovine species thus, in itself, determining degrees of expression of these candidate genes within the internal tissues sampled.

3.2.3.3 Statistical Analyses

The experiment was carried out as a randomized block. Animals receiving either the 1.2M or 2.2M feeding level in period one were crossed over to the opposite feeding level in period two. All data were analyzed by least squares analysis of variance using PROC GLM (SAS 1998). The model included the fixed effect of breed, feeding level, interactions of breed and feeding level and period. Where period was not significant only the first three parts of the model were retained. All data were analysed for correlation using PROC CORR (SAS 2002), sum of squares. Tissue-specific mRNA gene expressions were analyzed using PROC GLM sum of squares for the effect of breed, feeding level and interaction between breed and feeding level. Significance was reported at $p \leq 0.05$, and trends were reported with p values between 0.10 and 0.05.

3.3 Results

3.3.1 General Metabolic Responses

3.3.1.1 Digestibility

As expected animals on the higher intakes showed slight increases in the total fecal energy loss but not the energy losses in urine and methane gas. The overall mean fecal energy loss (across intake levels and breeds) was 25.3 % of the gross energy intake from the 90 % concentrate diet. An average of 2.7% of animal intake energy was lost in the urine and 4.3% was lost as methane gas. Therefore digestibility values were slightly lower at the higher feeding level (Table 3-1) and were not significantly different between breeds. Average DM digestibilities were 72.3 and 76.6% at 2.2M and 1.2M feeding levels, respectively, and 74.5, 74.7 and 74.2% in Angus, Brangus and Charolais, respectively. Average CP digestibilities were 80.8 and 88.3% at 2.2M and 1.2M feeding levels, respectively, and 84.8, 84.5 and 84.4% in Angus, Brangus and Charolais, respectively (Table 3-1). There was no interaction between breeds and feeding levels for DM, energy and CP digestibilities. The fecal, urinary and methane losses resulted in an average metabolizable energy content of the diet of 14.11 ± 0.71 $MJkg^{-1}$, which was approximately 67.7 % of the gross energy (Table 3-2).

Feeding a high concentrate diet usually results in a high digestibility in cattle. Lapierre et. al., (1992) reported that diet DM digestibility was decreased by increasing intake when steers were fed a 75% concentrate, pelleted diet at two intakes (low: 50 g/BW$^{.75}$ and high: 90 g/BW$^{.75}$ as fed), and similar results have also been reported by Wedegaertner and Johnson (1983). DM digestibility in the current experiment was 4.7 % lower at 2.2M than at 1.2M (72.1 vs 76.8). This is consistent with a decrease in digestibility of approximately 3%, which would have been predicted from ARC (1980) data. The average DE value of 74.7% over the entire experiment in the current study agrees closely with the DE of 75 % reported previously for Hereford heifers and steers fed a similar diet (Delfino et. al., 1988).

3.3.2 Breed Comparisons

The metabolizable energy intake (MEI) at which measured heat production (HP) are equal represents the metabolizable energy requirement for maintenance (MEm), and it was slightly, but not significantly, higher for the Charolais breed, in comparison to the other breeds. The slope of linear regressions of ER on MEI indicated that the efficiency of ME use for gain was not different for different breeds. Mean values were 0.58 for Brangus, 0.61 for Angus and 0.62 for Charolais and were not different. Estimates of maintenance (ME_m) of 0.49 $MJkg^{-0.75}d^{-1}$ for Angus and Brangus and 0.52 for Charolais were also not different.

3.3.3 Metabolic Response to Feeding

In the three breeds, heat production was consistently higher ($P < 0.05$) at the 2.2M intake compared to the 1.2M intake. Diurnal variations in heat production by the animals were substantial, especially in relation to feeding time. Statistical analysis showed that the percent increase in HP associated with feeding was higher; 23% ($P < 0.05$) at the 2.2M feeding level compared to 15% at the

1.2M feeding level. The differences ($P < 0.05$) in additional heat production (% of pre-feed) between 1.2M and 2.2M fed steers may have been due, in part, to differences in time spent eating and the energy requirement associated with feeding. Overall, the variation of additional heat production associated with feeding was small between breeds. The parameters related to efficiency measured were not markedly different between breed types, though the Charolais cattle had a tendency for a lower percent change in energy expenditure in response to feeding compared to the other breeds. Therefore the Charolais' adaptive response to feeding yields a result that is consistent with the slightly higher efficiency of ME utilization above maintenance for this breed.

3.3.4 Variation in Heat Production Among Individual Animals Within a Breed.

There was larger variation in heat production among individuals within a breed than between the different breeds. The coefficient of variation for HP among animals within each breed was 24.6%, 24.9% and 16.7% for Angus, Brangus and Charolais groups, respectively. It is possible that some of this variation may be linked to differences in gene expression. The study, therefore, provided us with the opportunity to examine relationships between energy expenditure and the relative expression of specific candidate genes.

3.3.5 Gene Expression

3.3.5.1 Gene Expression Study of UCP 1, UCP-2 and UCP-3

UCP-1 expression was found in trace amounts in mesenteric and subcutaneous adipose in some but not all animals. It can not be excluded that the sporadic UCP-1 observations within these white adipose depots were due to brown fat contamination within these white adipose samples, especially since we sampled from relatively young animals. Furthermore the pattern of UCP-1 mRNA expression was neither correlated with breed nor feeding level in this trial and thus will not be discussed further. In contrast, UCP 2 mRNA was detectable in most of the tissues screened and UCP-3 mRNA was observed in skeletal muscle, perirenal adipose and cardiac muscle (see Figures 3-1and 3-2).

The demonstration of UCP-3 message in bovine cardiac muscle is the first documentation of such presence in cattle (Figure 3-2). The expression of UCP-3 mRNA in cardiac muscle was found to be negatively correlated ($p= 0.04$, $r = -0.34$) with energy retention. No significant differences in UCP-3 gene expression profiles could be attributed to breed or feeding level alone and this was most probably due in part to the broad individual animal variations in this gene's expression profiles. Although UCP-2 mRNA was measured in many different tissues we found that individual variability within a breed was usually higher than differences between breeds with only a few exceptions. One exception that was observed pertained to the average UCP-2 mRNA levels present in Brangus rumen papillae which were found to be significantly lower ($p=0.006$) than observed in both Angus and Charolais. Secondly, when comparisons between UCP-2 mRNA in mesenteric adipose were made in relation to full (2.2X maintenance) and restricted feeding (1.2X maintenance), both the Angus and Brangus breeds showed higher levels in full fed animals ($p<0.001$) whereas UCP-2 mRNA expression in Charolais animals was not significantly affected by feeding level.

UCP-2 mRNA expression in skeletal muscle ($p<0.01$), subcutaneous and mesenteric adipose ($p<0.02$) were positively correlated to average daily gain in these steers. Furthermore multiple regression analyses showed that the expression of UCP-2 mRNA within several tissues of a given animal is also significantly related to its average daily gain ($r = 0.43$). Further, mesenteric adipose expression of UCP-2 mRNA is positively correlated with both heat production and metabolizable energy intake. A positive correlation between mesenteric adipose UCP-2 mRNA and MEI suggests that increased intake may increase the expression of UCP-2 in this tissue and, as expected, when nutrient availability increases so do the ADG and heat production. The UCP-2 mRNA in mixed fiber type skeletal muscle (*biceps femoris*) showed a similar significant increase in correlation with increased MEI ($r= 0.45$, $p<0.02$). Furthermore when multiple regression analysis was utilized to compare combined UCP-2 mRNA (UCP-2 combined) expression in mesenteric, subcutaneous, and perirenal adipose and biceps femoris relative to MEI a strong association was revealed ($r = 0.43$, $p<0.02$). Given that our measured average heat production increased in accordance with increased metabolizable energy intake (MEI), it was reasonable that our UCP-2 mRNA in mesenteric adipose and UCP-2 combined, were both positively correlated with the average heat production (Av. HP), $r=0.40$ and $r=0.33$, respectively. Our study further showed that UCP-2 mRNA in mesenteric adipose was strongly correlated with metabolizable energy retention (MER) values ($r=0.44$, $p<0.01$).

3.4 Discussion

In an early publication by Ferrell and Jenkins (1984), comparisons were made between various breeds of cattle and their ME of maintenance. Specifically, the maintenance requirements of mature non-lactating cows were compared and found to differ between four cross breeds of cattle; Angus cross, Charolais cross, Jersey cross and Simmental cross. Even prior to this, Ledger and Sayers (1977) found that plane of nutrition alters energy of maintenance requirements in cattle species. If these physiological attributes are consistently different between breeds then they must be heritable and as such be mediated by one or more genes. Three breeds were selected based on expected phenotypic variation and both metabolic parameters and candidate gene mRNA expression were measured for inter and intra-breed comparison. Our examination of energy utilization in representative cattle for three breeds of cattle, on two-planes of nutrition revealed some interesting correlations. Though we perceived that physical stature differences between the breeds selected would translate into similar differences in MEm observed, as those of previous studies, the breeds in the current study were not significantly different in this regard. The Charolais animals showed a non-significant trend for slightly higher maintenance as would be expected by its larger stature. It should be noted that our cattle were still growing and thus had not yet reached the maximum expected size disparity between animals of these different breeds and, as such, it is possible that the trend observed may have reached significance if measurements had been extended to later stages of animal maturity. Further regression analyses of energy retained (ER) versus metabolizable energy intake (MEI) did not reveal any significant breed differences in efficiency of ME used for gain. A more detailed review of these findings relating to measures of efficiency and digestibilities in these breeds are included in two separate submissions of these results (Moibi et. al., unpublished; Li et. al., unpublished).

Evaluation of uncoupling protein mRNA expression in various tissues of these breeds was performed to ascertain breed differences, if any, as well as to determine whether any observed mRNA expression differences were related to measures of energetic efficiency. Although investigation of the relationship between energy expenditure and UCP gene expression has been performed in rats, mice and humans there has been limited investigation of these genes in livestock species. Only UCP-1 had been previously characterized in any detail in cattle and expression of UCP-2 and UCP-3 remained in relative research infancy prior to the current study in beef cattle. Since it is understood that heat production in animals is in large part derived from the inefficiencies associated with the biological metabolic conversion of nutrients into anabolic substrates and especially the cycling of ATP; the putative uncoupling of the mitochondrial proton gradient and ATP production mediated by UCP's would be expected to increase heat production. UCP-2 has been shown to retain the capacity to uncouple, when expressed in vitro in yeast and other mammalian cells (Fleury et. al., 1997; Ricquier and Bouillaud, 2000). Furthermore, a study of 640 French Canadians predisposed to weight gain reported linkage between three microsatellite markers that encompass the UCP-2 gene location (in humans 11q13), with resting metabolic rate, body mass, percentage body fat, and overall fat mass (Bouchard et. al., 1997). Additional support for a role of UCP-2 in adaptive thermogenesis is conveyed by its consistent up-regulation in response to cold exposure observed in a broad range of studies, including those in chickens (Raimbault et. al., 2001), hummingbirds (Vianna et. al., 2001), and even a UCP homologue in plants (Pecquer et. al., 2001). It was somewhat surprising that when UCP-2 knockout mice were established they were neither obese nor cold-sensitive, but this was in part explained by an observed up-regulation of white adipose UCP-1 expression inversely proportional to measured UCP-2 levels in knockout mice (Arsenijevic et. al, 2000). This same group did not observe any similar compensatory up-regulation of UCP-3 in these UCP-2 knockout mice.

UCP-3 also appears to retain the capacity to uncouple as demonstrated by both in vitro liposome proton transport studies (Jaburek et. al., 1999) and the in vivo observation that mice lacking skeletal muscle UCP-3 are more coupled than their wild-type counterparts (Vidal-Puig et. al., 2000). Further, transgenic mice that over express human UCP-3 in their skeletal muscle are hyperphagic while having a striking reduction in body mass and specifically white adipose stores (Clapham et. al., 2000). An earlier study in mice demonstrated that UCP-3 expression was induced upon exposure to several known thermogenic stimuli including β3-adrenergic agonists, thyroid hormone and leptin (Gong et. al, 1997). When UCP-3 knockout mice were established and examined they were found to be of normal body-weight and retained normal levels of cold tolerance (Vidal-Puig et. al, 2000). Although other UCP isoforms were not noticeably up-regulated in this instance, alternative mechanisms of compensating for the loss of UCP-3 may be responsible for the apparently normal body weight and cold-tolerant phenotype.

In the cattle in the current study, UCP-2 mRNA remained the most ubiquitously expressed subtype of uncoupling protein as it was present in all tissues examined except abomasum and duodenum. In contrast, UCP-3 mRNA was detectable in only three of our tissues, *biceps femoris* skeletal muscle, perirenal adipose tissue and notably cardiac muscle. Although UCP-3 is predominantly expressed in skeletal muscle (Vidal-Puig et. al, 1997), its expression in other metabolically important tissues such as adipose (perirenal) tissue and cardiac muscle may be indicative of its importance in adaptive thermogenesis and/or moderation of reactive oxygen species accumulation in tissues with higher metabolic rates. The observed uncoupling protein mRNA expression profiles in the cattle tissues in this study were similar to those patterns observed in tissues of other animal species, and this will facilitate our comparison of research performed in other species with what may be examined, and related in cattle. The measurement of UCP-2 mRNA across our experimental samples, reveals fewer than expected differences in expression profiles correlated to either feeding level treatment or between breeds, though some differences were observed and will be discussed later in this section. Moreover, what is important to realize is that the large standard deviations for our UCP-2 and UCP-3 mRNA measurements illustrate the variability between individual animal's expression, which was typical both within and between animals from any of our breeds examined.

Other studies indicate that expression of UCP-2 is influenced by catecholamines such as norepinephrine (Thomas and Palmiter, 1997), thyroid hormones (Guerra et. al, 1996 & Obregon et. al., 1996), peroxisome proliferation activating receptors (PPAR's) (Aubert et. al., 1997) and leptin (Zhou et. al. 1997), all of which are known to vary substantially between individuals as well as under differing physiological conditions. Given that so many factors are known to mediate the expression of UCP, it supports the hypotheses that finite regulation of this gene is important and pertinent to individual animal and tissue metabolic status. The capacity of UCP-2 to be up-regulated by catecholamines, known to be elevated during conditions of stress such as cold exposure, in conjunction with the varied level of expression of UCP-2 over numerous tissue types add some level of support to hypotheses that this gene may be important in adaptive thermogenesis and other adrenergically-mediated regulatory pathways. It is possible that UCP-2 mRNA expression in cattle may be influenced by fasting as is observed in humans (Millet et. al., 1997), and that our lower level of intake (1.2X maintenance) was insufficient in terms of nutrient restriction to induce similar changes in the expression profile. Although speculative, the variability observed in the UCP-2 gene expression between individuals within and between breeds may ultimately address some of the physiology behind similar variabilities observed in individual animal energetic efficiencies. Further, variablity in parameters related to nutrient intake and heat production may also be related to the various tissues UCP-2 mRNA expression, some of which will be discussed in subsequent paragraphs.

The expression of the UCP-3 gene is much more restricted across tissues than that of UCP-2, however its expression in important metabolic tissues such as skeletal and cardiac muscle as well as peri-renal adipose tissue suggests that this gene may contribute to whole animal metabolism and efficiency. Although we did not observe any significant differences in UCP-3 mRNA solely attributable to either breed or the two levels of feed intake examined, we cannot preclude that such differences could not exist between other cattle under more extreme nutrient disparity. The expression of UCP-3 gene in skeletal muscle, cardiac muscle, and perirenal adipose again showed substantial individual animal variability (Table3-4 and 3-5). Individual variability was proportionally less in cardiac muscle than in the mixed fiber type skeletal muscle (biceps femoris) but it is as yet unknown whether this is consistent over other skeletal muscle types. Skeletal muscle is typically considered the primary site of expression for UCP-3 (Boss et. al., 1997) and, as such, the majority of research to date has focused on this tissue type. UCP-3, has been shown to be regulated in rat skeletal muscle by thyroid hormone (Larkin et. al., 1997), leptin (Liu et. al., 1998) and catecholamines (Gong et. al., 1997). It is apparent that skeletal muscle is a suitable target tissue for investigating varied efficiencies in cattle since not only is it the marketable product that can vary between individual animals in terms of quality but it also represents as much as 50% of the live animal weight and as such this tissue's metabolism represents a significant proportion of the whole animal's metabolism.

Furthermore, it is known that skeletal muscle is very metabolically active and dynamic under varied conditions, be they catabolic or anabolic states.

The detection and measurement of UCP-3 in bovine cardiac muscle is the first such report but is supported by observation expression in both of this gene rats and humans. It is very interesting that UCP-3 mRNA expression was negatively correlated to energy retention (r = -0.34). It is known that treatment of rats with thyroid hormone elevates their metabolic rate and increases cardiac muscle UCP-2 and UCP-3 mRNA levels (Degens et. al., 2003). If cardiac muscle UCP-3 mRNA levels are under considerable influence of thyroid hormone levels in cattle, then it stands to reason that animals with higher thyroid activity would have higher systemic metabolic rates, higher cardiac muscle UCP-3 mRNA levels and would retain less of their nutrient intake as body mass. Thus energy retention would be negatively related to cardiac muscle UCP-3 expression.

UCP-3 mRNA has been observed in piglet skeletal muscle and adipose, as reported by Damon et. al., (2000), and it is further implicated in terms of affecting the energy metabolism of rats under fasting states. The lack of associative changes in UCP-3 expression levels with our reduced feeding level is most probably a reflection of the modest degree in restriction that our treatment represents, but may also have been influenced by our time of sampling which may have missed some acute regulatory responses.

Analyses of UCP-2 mRNA expression in the rumen papillae of the three breeds examined in the current study indicated the Brangus cattle expressed a lower relative amount of this message than was observed in either Angus or

Charolais steers. This is an important though preliminary observation and may suggest varied efficiencies in ruminal tissues of animals of different breed on similar diets. Further analyses of UCP-2 mRNA expression in rumen papillae sampled over more frequent and acute time periods in additional breeds under more varied conditions are required to validate the relevance and importance of this observed difference between breeds. Another breed difference in UCP-2 mRNA expression, this time in mesenteric adipose, was related to feeding level. Whereas both Angus and Brangus cattle showed significantly elevated UCP-2 mRNA in full-fed (2.2X) animals, UCP-2 mRNA expression in this tissue in the Charolais steers were not significantly different at the two feeding levels. It is important to mention that although not accurately measured in the study, a subjective interpretation was that overall the Charolais steers in our study were of leaner body mass than the steers of the other breeds. Given that the Charolais steers were leaner, then the metabolic status of their mesenteric adipose depots could have been different at the postmortem sampling point than other steers that may have been actively storing more fat in this adipose depot. Once again this apparent difference observed in breeds on different nutrient planes will be better understood upon re-examination of UCP-2 mRNA in this tissue under additional feeding levels and time points as well as in steers in which a less subjective determination of adiposity could be obtained.

Comparison of gene expression profiles over the animals with measured indices of efficiency and heat production reveals some important and significant correlations (Table 3-4), although it must be emphasized that correlations do not determine either cause or effect and should only be made to reference associations based on sound hypotheses. The expression of UCP-2 mRNA in skeletal muscle, subcutaneous adipose tissue and mesenteric adipose tissue are all positively correlated with ADG. When multiple regression was applied, the combined expression of UCP-2 mRNA in skeletal muscle, peri-renal adipose tissue, mesenteric adipose tissue and subcutaneous adipose tissue there was a significant correlation ($r = 0.43$) to the measured ADG over the entire period. Given that the uncoupling proteins are believed to uncouple the mitochondrial proton gradient from ATP synthesis, hence reducing efficiency of energy conversion, this positive association with body weight gain may seem surprising. It is possible that the expression levels of UCP-2 mRNA may be indicative and representative of the nutrient availability in these tissues. Given such a hypothesis, animals that show higher rates of average daily gain may represent animals that have improved capacity to partition their nutrients to anabolic tissue sites such as the skeletal muscle and subcutaneous and mesenteric adipose. These same animals may also systemically adapt to express higher levels of uncoupling proteins on the basis of ultimately having higher established and more persistent proton gradients within their mitochondria associated with greater energy retention. This may also be supported by the positive correlation($r = 0.44$) between UCP-2 expression in mesenteric adipose and MEI (MJ/kg bw 0.75). In this instance the expression of UCP-2 may be a useful marker for growing animals that inherently show better degrees of body weight gain on the same nutritional plane. A modest though significant correlation ($r = 0.33$) was observed between the expression profile of

UCP-2 mRNA in mesenteric adipose and heat production across all animals regardless of treatment. This correlation might be more attributable to the capacity of UCP-2 to uncouple the oxidative phosphorylation pathway from energy storage in terms of ATP synthesis. If we consider that the mesenteric adipose depot represents the long-term storage of lipids that accumulate in an animal provided with sufficient and/or abundant nutrients for growth, then the uncoupling of ATP synthesis in these animals should result in increased heat production. Support for this concept is provided by the demonstration that UCP is up-regulated by the presence of intracellular fatty acids (Klingenberg, 1993), as would be the case in nutrient abundance in cattle. The results of the current study show that UCP-2 mRNA expression in the mesenteric adipose ($r = 0.46$) was positively correlated to MEI, supporting the theory that increased nutrient availability in this adipose tissue is associated with an up-regulation of the UCP-2 mRNA expression. In other words the greater expression levels of UCP-2 in mesenteric adipose could result in increased energy release in the form of heat rather than ATP synthesis, and this would be observed as an overall elevation in whole animal heat production assuming that all other factors are equal. This correlation represents the first such evidence of a link between whole animal heat production and uncoupling protein-2 expression in bovine species. Moreover, the study showed that UCP-2 mRNA expression in mesenteric adipose was significantly correlated with a steers energy retention ($r = 0.44$), which may further support the hypothesis that nutrient abundance and increased anabolic fat storage in the mesenteric depot is associated with increased UCP-2 mRNA transcription. Further and in contrast, a significant negative correlation (r=-0.34) was observed between the expression of UCP-3 in cardiac muscle and energy retention, which could suggest that the expression of this gene in the heart is inversely associated with an animal's whole body energy retention. It would not be surprising to observe opposing adaptational responses in adipose depots and cardiac muscle in terms of their tissue specific metabolism as it is expected that vital core organs such as the heart are regulated diffeently than nutrient-storing tissues. Research in humans by Cortright et. al., (1999), has demonstrated a positive correlation between UCP-3 gene expression in muscle and exercise, which may support the interpretation that elevations in metabolic rate, as are typical in exercise, result in elevated UCP-3 and reduced energy storage or retention. If it is assumed that cardiac fitness is related to whole animal fitness, then reduced energy retention may indicate reduced sedentary behaviour or in effect increased energy expenditure, and the elevation of UCP-3 in cardiac muscle may be representative of these more active albeit less efficient cattle.

Additionally, a trend, though not quite significant with our degrees of freedom (r = 0.29) for a positive correlation between liver UCP-2 expression and efficiency of energy retention, was observed. The efficiency of energy retention may thus be an indicator of the level of nutrient exposure in the liver. If more nutrient availability in the liver elevates the observed UCP-2 mRNA expression as a result of elevated and/or more persistent proton gradients in liver mitochondria, then this association may be reflect, rather than contribute to an observed increase in efficiency.

The characterization of UCP-2 and UCP-3 gene expression initiated in this study in bovine species may assist with future capitalization on the research performed on this gene in other species as well as provide a future reference for additional study of this gene in cattle. Given the variations between individual animal expression of the UCP-2 gene, especially in the skeletal muscle, adipose depots and liver, further studies addressing more acute sampling and analyses of these tissue types may be essential in order to understand the underlying cause(s) of the observed variations. The results of our study highlight the importance of examining the expression patterns of genes that are suitable candidates for influencing energetic efficiency, nutrient processing, nutrient partitioning and growth. The objective of understanding the components and interactions of complex physiological systems that regulate the processes of nutrient conversion into energy for maintenance and growth will ultimately facilitate efficient production and breeding practices towards obtaining, and selecting for, the most desirable attributes specific to each region of livestock production. The results of this study showed that UCP-2 and UCP-3 mRNA expression levels were variable within animals of a similar breed, at least in the breeds that were examined, and in some cases appeared to vary between breeds utilized in this research. It must be emphasized that, in spite of our use of 12 animals per breed selected, these numbers are too small to assume that they absolutely represent their respective breeds. It remains possible that further examination of these and other breeds may identify some important breed differences, even with respect to UCP-2 and UCP-3 expression. Moreover, the expression of these isoforms of uncoupling protein, show important differences in tissue-specific expression that relate to the adaptive homeorrhetic state of those tissues. One benchmark of useful research is that the results not only yield insightful and descriptive information but also that the results stimulate additional related questions to be resolved by subsequent studies. As this study provides the first significant examination of UCP tissue-specific mRNA expression and it's relationship to MEI, HP, MER as well as feeding level and breed in beef cattle, it provides the framework that will facilitate identification of the physiological function of these isoforms in ruminants. The relationships determined between these genes and various indices of energetic efficiency substantiate them as good candidates for genes that play a physiological role in mediating tissue-specific processes that may effect whole animal metabolism and thus be relevant to livestock production.

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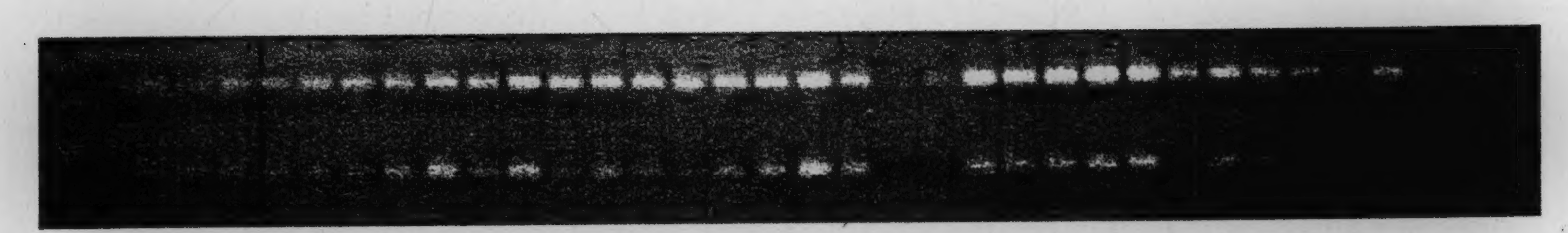

Figure 3-1 Image of Cardiac muscle UCP-2 amplicon (263 b.p. bottom) with G3PDH internal control (480b.p. top) separation on 2% (w/v) agarose gel. Each lane represents an individual animal.

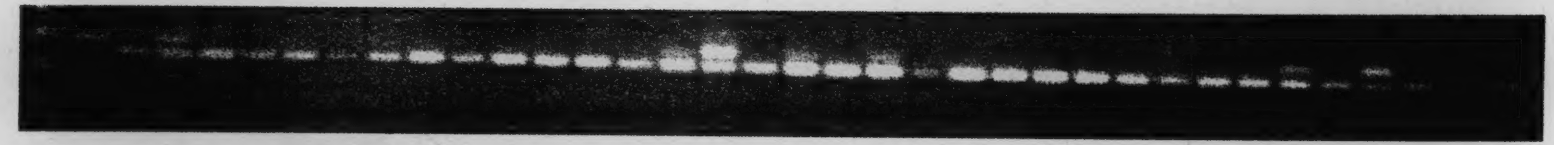

Figure 3-2 Image of Cardiac muscle UCP-3 amplicon (499b.p. top) with G3PDH internal control (480b.p. bottom) separation on 2% (w/v) agarose gel. . Each lane represents an individual animal.

Table 3-1 Breed comparison of Mean Percent of intake for Digestibility[Z] of DM, and protein at two levels of feeding above maintenance.

	DM Digestibility		Protein Digestibility	
Breed	1.2M[*]	2.2M	1.2M	2.2M
Angus	76.58 ± 3.74	72.448 ± 3.74	88.460 ± 1.61	82.136 ± 1.61
Brangus[†]	75.60 ± 4.69	73.725 ± 4.69	87.778 ± 2.02	81.156 ± 2.02
Charolais	77.58 ± 4.23	76.540 ± 4.23	88.620 ± 1.82	82.754 1.82

Notes: [z] expressed in mean ± pooled breed SE.
[*] 1.2 x or 2.2 x estimated requirement for maintenance.

Table 3-2 Comparison of UCP-2 mRNA expression[y] in three internal tissues as observed in three breeds.

	UCP2 Liver	UCP2 Rumen papillae	UCP2 CM[x]
Breed	lsmean	lsmean	lsmean
Angus	53.50±15.35	725.92± 19.99	814.33±11.04
Brangus[†]	56.46±15.35	**638.73± 19.99**	800.32±11.04
Charolais	56.50±15.35	722.26± 19.99	792.63±11.04

Notes: [y]arbitrary densitometric units,[z] expressed in mean ± pooled SE.

[x] Cardiac muscle

Table 3-3 Mean UCP-2 mRNA abundance [y] in adipose tissues comparisons between three breeds and two levels of feeding above maintenance.

	MA[w]		SA[v]		BF[u]	
Breed	1.2M	2.2M	1.2M	2.2M	1.2M	2.2M
Angus	500.0±25.8	**620.2±25.8**	452.7±24.9	461.9±24.9	67.5±18.9	71.9±18.9
Brangus[†]	506.6±25.8	**553.2±25.8**	421.2±24.9	447.9±24.9	99.1±18.9	76.9±18.9
Charolais	543.5±25.8	529.9±25.8	454.0±24.9	430.7±24.9	82.11±18.9	101.3±18.9

Notes: [y] arbitrary densitometric units, [z] expressed in mean ± pooled SE.

[w] Mesenteric adipose tissue, [v] Subcutaneous adipose tissue, [u] *biceps femoris* skeletal muscle

Table.3-4 Correlations of tissue- specific UCP-2 and UCP-3 mRNA abundance^z with measures of energetic efficiency and heat production

	UCP-2 Biceps femoris	**UCP-3 Biceps femoris**	**UCP-2 subcu adipose**	**UCP-2 mesenteric adipose**	**UCP-2 perirenal adipose**	**UCP-3 perirenal adipose**	**UCP-2 rumen**	**UCP-2 cardiac muscle**	**UCP-3 cardiac muscle**	**UCP-2 liver**
Variable										
Heat production	-0.08	0.11	0.00	**0.33**	0.16	0.10	0.07	0.10	-0.09	-0.18
ADG (g/kg.75)	**0.45**	**0.34**	**0.31**	**0.44**	0.02	0.04	-0.01	0.19	-0.15	-0.04
MEI (MJ/kgbw.75)	0.15	0.17	0.12	**0.46**	-0.01	-0.11	0.16	0.01	-0.29	-0.16
MER	0.16	0.20	0.11	**0.44**	-0.12	-0.10	0.13	-0.01	**-0.34**	-0.15
DMER/DMEI	0.07	-0.09	0.17	0.05	-0.10	-0.14	0.04	0.07	-0.02	**0.29**
ME maintenance	-0.02	-0.22	0.19	0.05	0.23	0.00	-0.10	0.18	0.06	**0.28**

n = 35.

^{z} arbitrary densitometric units

Table 3-5 Effect of breed and feeding level on mRNA expression^z of UCP2 and UCP-3 in tissues.

	UCP-2 Biceps femoris	UCP-3 Biceps femoris	UCP-2 subcu adipose	UCP-2 mesenteric adipose	UCP-2 perirenal adipose	UCP-3 perirenal adipose	UCP-2 rumen	UCP-2 cardiac muscle	UCP-3 cardiac muscle	UCP-2 liver
Treatment										
2.2X	84.0	135.0	445.9	562.9	161.8	606.9	706.3	798.2	587.6	51.8
SD	46.3	20.4	64.0	69.4	51.7	32.6	59.5	32.8	25.0	41.4
1.2X	82.9	135.5	442.7	517.0	159.1	602.5	682.8	806.1	603.1	59.8
SD	37.2	23.9	53.8	62.4	58.7	14.5	92.0	40.9	40.5	57.9
Breed										
Angus	67.05	139.4	445.8	547.6	138.5	599.5	712.9	811.3	598.9	54.0
Brahman-Angus	92.42	138.2	444.6	535.8	174.5	614.2	643.3	802.8	596.3	57.5
Charolais	91.7	128.3	442.4	532.2	169.5	601.1	722.2	792.6	591.6	56.5
SD_{total}	41.3	21.9	58.1	68.9	54.6	24.7	77.7	36.9	34.3	50.0

^{z} arbitrary densitometric

CHAPTER 4

The mRNA Expression of Leptin, Neuropeptide Y and Receptors in Bovine Tissues and Their Relationship to Breed, Feeding Level and Measurements of Energetic Efficiency

4.1 Introduction

As many studies have previously determined, feed intake is perhaps the single most important factor affecting the growth of cattle, which makes intuitive sense since growth requires the ingestion of nutrient building blocks for anabolic processes. Of course, other factors influence growth including animal health, environment and individual animal genetics and physiology. It is generally considered that nutrient intake is under the control of the producer, and their provision of feed, but we also are well aware of an individual animal's dependence upon systemic orectic and satiety signaling to mediate intake (as reviewed by Lawrence et. al., 1998 and Woods et. al., 1998). Although several different physiological orectic and anorectic mediators are known, this study will focus upon the tissue-specific expression of message for the satiety hormone (leptin), the ingestion promoting hormone (neuropeptide Y (NPY)) and their respective receptors which mediate the tissue-specific responses to these circulating peptides.

Leptin, a 167 amino acid peptide, is the product of the OB gene which was originally cloned from rats and humans by Zhang et. al., (1994), and subsequently in cattle by Ji et. al., (1998). More than 40 years earlier, Kennedy (1953) suggested that the stability of whole animal adiposity must be regulated by a lipostat that signals in response to the amount of body fat stores to influence the animal's food intake and energy balance. Leptin is synthesized and secreted primarily from white adipose tissue (Banks et. al., 1996) and is transported in the blood to its effector organs that are characterized by their expression of it's specific cell surface receptor (a product of the db gene). Inducible leptin expression has also been reported in skeletal muscle (Wang et. al., 1998) and in the rat and human stomach (Bado et. al., 1998, Sobhani et. al., 1999).

The exogenous administration of leptin to animals results in a dose-dependent reduction in food intake, body weight, and fat depots with an increase in energy metabolism (Campfield et. al., 1995; Pelleymounter et. al., 1995; Chen et. al.; 1996; Levin et. al., 1996). These studies emphasize the importance of leptin in the systemic regulation of energy balance. Leptin has central effects moderated through the hypothalamus to reduce feed intake, and peripheral effects, at various tissues that express the leptin receptor, to increase metabolic rate, increase oxygen consumption and increase lipolysis (Hossner et. al., 1998). Leptin's effects on animal energy metabolism are, in general, at odds with efficient production, but an *in* vivo study in cattle has shown that adipose leptin mRNA expression is increased by exogenous growth hormone administration and thus may also be associated with lean body growth in steers (Houseknecht et. al., 2000). In a recent study it was shown that plasma leptin levels in cattle are positively regulated by energy intake, and relate to both body fatness and adipose cell size (Delavaud et. al., 2002, Minton et. al., 1998). The central activity of leptin is known to involve its ability to inhibit neuropeptide Y (NPY) synthesis in the hypothalamic arcuate nucleus (Stephens et. al., 1995; Schwartz et. al., 1996). In lactating ewes that were experiencing negative energy balance despite increased food intake, adipose depot leptin mRNA expression was found to be decreased and this may represent the sheep's attempt to maintain energy balance through hyperphagia (Sorensen et. al., 2002).

As mentioned earlier, at least part of leptin's central satiety signaling involves inhibition of hypothalamic NPY appetite induction pathways. NPY is a 36 amino acid peptide that arises through several post-translational processing steps of the original NPY gene product "pre-pro-NPY" that is 97 amino acids long through a 69 amino acid intermediate; "pro-NPY". NPY is primarily produced in the arcuate nucleus of the hypothalamus and is orectogenic, that is it promotes nutrient intake through induction of appetite. Prolonged central administration of exogenous NPY has been shown to promote adipose accumulation and ultimately result in obesity (Stanley et. al., 1986; Zarjevski et. al., 1993). Additionally, the post-prandial increase in both insulin and leptin are negatively correlated with hypothalamic NPY expression (Schwartz et. al., 1996). Sorensen et. al., (2002) found that lactating ewes not only showed decreased leptin mRNA but also elevated hypothalamic NPY which would also support hyperphagia necessary for the maintenance of milk production while minimizing negative energy balance. Leptin and NPY appear to be important components in the central regulation of energy balance via their opposing influence on nutrient intake. In conjunction with the analyses of these peptides in relation to energy balance in cattle, their respective receptor mRNA expression was evaluated in various tissues to test the hypothesis that the expression of the receptor genes (leptin and NPY) within tissues represents the set point of responsiveness of an animal to the influences of these endocrine peptide ligands. Support for this idea comes from several studies. A mutation in the db gene in mice, that encodes the leptin receptor, results in leptin insensitivity and phenotypic obesity (Tartaglia et. al., 1995). It has also been shown that increased leptin receptor mRNA in the hypothalamus is associated with leptin hypersensitivity and associated fasting response (Baskin et. al., 1998) whereas, NPY receptor mRNA is increased in association with scenarios of hyperphagia, and further in animals with concurrent development of leptin resistance and body weight gain (Kalra et al., 1998)

An attempt was made to characterize the mRNA expression of these genes, in steers of different breeds at two different feeding levels, while determining if the level of gene expression can be correlated with metabolic status of cattle and hence be related to indices of metabolic rate and/or energy conversion.

4.2 Material and Methods

4.2.1 Animals and Treatments

Thirty-six cattle, 12 each of three breed types (15-17 months); Charolais {503.7kg +/- 40.3kg}, Angus {459.3kg +/- 37.6kg}, and Brahman/Angus cross {405.9kg +/- 24.9kg} were utilized in this experiment. These cattle were maintained at the University of Alberta Environmental and Metabolic Research Unit, Edmonton Research Station in accordance with FAPWC and UAPWC as being in accordance with CCAC guidelines (CCAC, 2003). The selection of breeds was designed to lead to a higher probability of measured differences, due to differences in size and genetic and phenotypic attributes. However these animals were acquired from individual herds and, as such, are representative of similar animals but can not be considered as absolute references of their given breeds. Starting at 15-16 months of age, the thirty-six steers (Charolais, Angus and Brangus) were fed either restricted (1.2X maintenance) or high (2.2X maintenance) intake levels of a standard feedlot diet (approximately 90% (w/w) Barley conc., 10% (w/w) roughage, this mixed diet contained, on average, ME 14.496 MJ/kg of DM; CP 14.93 % (w/w) DM, for two periods of 45 days each in a crossover design. Diet ME values were estimated and prepared for each animal initially using NRC (1984) feed guidelines to establish intakes. Animals were individually housed (18 ± 2 °C room temperature) in floor pens (3 m x 3 m) with wood shavings as bedding. Feed was offered to the animals between 0800 and 0900 h daily and orts were collected and weighed. Prior to the commencement of the study the steers were fed in groups and had constant access to water throughout the adaptation period. Animals were weighed before they were placed on the treatments and at two week intervals. Before the start of period one, animals were gradually introduced to the concentrate diet, and were allowed a further 2-3 weeks of adaptation once the 2.2M groups achieved satisfactory intake. After the completion of period one, feed supply to the 1.2M animals that were being transferred to 2.2M was gradually increased while those from 2.2M in period one was gradually brought down to 1.2M. The adaptation length was similar in all periods but varied slightly by animal according to the order in which they were placed in calorimetry hoods. Energy expenditure measurements (respiration calorimetry) were made during the last 10 days of each period.

While in the respiration hoods the animals had access to both feed and water although their mobility was limited (to standing, lying, feeding, and drinking) by both the respiration hoods and stanchions. Additionally, after dietary acclimation, biopsy samples of the *biceps femoris* muscle (0.5-1.0g) were taken from each of the treatment animals, within the few days immediately following calorimetry measurements. Total RNA was isolated from each tissue for assessment of gene expression using RT- PCR and semi-quantification of the specific cDNA amplicons. After period 2, animals were kept on their respective feeding levels up until the day of slaughter. Samples of muscle, adipose tissue and selected internal organs (liver, heart, duodenum, abomasum and rumen papillae) were collected, at slaughter, for isolation of RNA to study gene expression in conjunction with the metabolism measurements (detailed protocols for biopsy and post-mortem tissue collection are included in the Appendices).

4.2.2 Assessment of Energy Utilization

Feed intakes were measured daily from the start of adaptation to the end of each experiment period. Fecal excretion of energy and nitrogen were determined by reference to lignin and acid-insoluble ash in feed and fecal grab samples collected over a three-day collection during each period. Urinary excretion of energy, and nitrogen were determined during three days of total urine collection.

Detailed methodology for calorimetry, digestibility, urine collection and laboratory analyses is described in Appendix one.

Heat production (energy expenditure) was recorded on individual animals using respiration calorimetry while the animals were fed on different planes of nutrition (estimated 1.2X and 2.2X maintenance using NRC guideline). These data provide estimates of energy retention, maintenance requirement and efficiency of utilization of retained energy and of protein retention. At slaughter, data was obtained on grade and fat.

4.2.3 Gene Expression Studies

4.2.3.1 RNA Extraction

Total RNA was extracted from measured quantities of frozen powdered tissue samples for each individual animal using a modified Chomczynski protocol (1993) and Gibco Trizol™ phenol-chloroform phase extraction procedure. The integrity of each Total RNA sample was examined using electrophoresis on agarose gels and examination of the abundance and integrity of the 18S and 28 S ribosomal RNA bands. The total RNA purity and quantity were determined for each sample using absorbance measurements at wavelengths of 260nm and 280nm, and the respective ratio of these measures (quantification calculations are shown in the appendix). Working stocks (50μl) of 1μg total RNA/μl for each sample were prepared using DEPC-treated water. These working stocks were utilized for the reverse transcription and subsequent PCR analyses.

4.2.3.2 Reverse Transcription and Polymerase Chain Reaction

Reverse transcription was performed for each sample using 1ug total RNA, oligo-dT^{15} primer and Gibco MMLV-RT enzyme, in the presence of RNase inhibitor (Gibco RNAase OUT) at 37 ° C for 60 minutes in a thermocycler (Perkin Elmer 2400 or BioRad Icycler). Additional details regarding the reverse transcription protocol are included in the Appendix. Each reverse transcription reaction supplied template for a minimum of three specific multiplex PCR reactions using specific primer sets and Taq polymerase enzyme (Sigma). Sense and antisense primer sets were designed for each of the specific target genes using Genbank as a resource for specific bovine gene and mRNA sequences. Genejockey and Amplify software were utilized to design and conceptually test each of the primer sets. The inherent principal of primer design is such that if sense and antisense primer sets are created that have 100% sequence homology to a known gene's messenger RNA and, given that each primer is about 20 bases long, annealing of such primers during PCR is specific and preferential to the intended gene's mRNA and as such will result in amplification of only that message. Our optimized primer sets are able to specifically amplify and thus semi-quantify bovine mRNA representing the following genes; leptin, neuropeptide Y (NPY), leptin receptor (Lrec) and neuropeptide Y receptor (Nrec). Further, we have utilized primer sets for constitutively expressed genes; either glyceraldehyde-3-phosphate dehydrogenase (G3PDH) or cyclophilin, as our internal control in each of our specific relative reverse transcription polymerase chain reactions (relative RT-PCR). This internal control normalizes for slight variations in reaction efficiency, RNA loading and pipetting. All PCR reactions were performed on either a Perkin Elmer 2400 thermocycler or BioRad Icycler. The products of the PCR reactions (amplicons) were electrophoresed on a 2%

(w/v) agarose /TBE gel along side a molecular weight marker (Gibco 100bp DNA ladder). The gels were stained using ethidium bromide (0.1% w/v) so as to observe the PCR products under UV fluorescence. The specific amplicon bands could thus be relatively quantified generating optical density scores for the amplicon bands using BioRad's Gel Doc and Molecular Analyst software.

Expression of messenger ribonucleic acid (mRNA) for specific candidate genes was measured by relative RT-PCR analyses using primer sets designed with homology to known bovine gene sequences. These primers were designed to include a trans-intronic region of the gene sequence so that the mRNA amplicon would definitively be smaller than an amplicon generated from genomic DNA contamination. The RT-PCR reactions were validated for linearity with regards to total RNA quantity and cycle number and the amplicons were sequenced to validate the specificity and selectivity for the desired uncoupling protein isoform. Comparisons of relative gene expression for individual animals could thus be related to other parameters including measured indices of metabolic rate such as indirect calorimetry heat production and indices of intake and efficiency such as metabolizable energy and energy retention values. Through our cross-over design we compared gene expression profiles of these candidates under the influence of the two different feeding levels in *biceps femoris* skeletal muscle. Additionally we examined mRNA expression differences within and between three breeds of cattle to determine to what extent apparent variations in efficiency were related to individual gene expression of these selected genes in the metabolically important tissues sampled. Further, analyses of these candidate genes in the various tissues collected *postmortem* allow some comparisons to be made between the different gene expression profiles over several metabolically important tissue types. Moreover, this study represents the measurement of many of these candidate genes and their tissue expression profiles for the first time in bovine species thus, in itself, determining new knowledge concerning mRNA for these genes

4.2.2.3.3 Statistical Analyses

The experiment was carried out as a randomized block. Animals receiving either the 1.2M or 2.2M feeding level in period one were subsequently crossed over to the opposite feeding level in period two. All data were analyzed by least squares analysis of variance using PROC GLM (SAS 1998). The model included the fixed effect of breed, feeding level, interactions of breed and feeding level and period. Where period was not significant only the first three parts of the model were retained. All data were analyzed using PROC CORR and sum of squares (SAS 2002). Significance was reported when p values were less than or equal to 0.05, trends were reported when pvalues were between 0.05 and 0.10.

4.3 Results

4.3.1 Metabolic Data

4.3.1.1 Digestibility

Animals on the higher intakes (2.2X) showed slight increases in total fecal energy loss but not energy losses in urine and methane gas. The overall mean fecal energy loss (across intake levels and breeds) was 25.3 % of the gross energy intake from the 90 % (w/w) concentrate diet. An average of 2.7% of animal intake energy was lost in the urine and 4.3% was lost as methane gas. Average DM digestibilities were 72.3 and 76.6% at 2.2M and 1.2M feeding levels, respectively, and 74.5, 74.7 and 74.2% in Angus, Brangus and Charolais, respectively. (Data reported in Li et al, to be submitted). Average CP digestibilities were 80.8 and 88.3% at 2.2M and 1.2M feeding levels, respectively, and 84.8, 84.5 and 84.4% in Angus, Brangus and Charolais, respectively. There was no interaction between breeds and feeding levels for DM, energy and CP digestibilities. The fecal, urinary and methane losses resulted in an average metabolizable energy content of the diet of 14.11 ± 0.71 $MJkg^{-1}$, which was approximately 67.7 % of the gross energy.

DM digestibility in the current experiment was 4.7% lower at 2.2M than at 1.2M (72.1 vs 76.8), which is consistent with a decrease of approximately 3%, in digestibility as predicted from ARC (1980) data. Our average DE value of 74.7% over the entire experiment in the current study agrees closely with the DE of 75 % reported previously for Hereford heifers and steers fed a similar diet (Delfino et. al., 1988).

4.3.2 Breed Comparisons

The metabolizable energy intake (MEI) that equals measured heat production (HP), measured at or near maintenance, represents the metabolizable energy requirement for maintenance (MEm). The MEI for our Charolais breed was slightly, but not significantly higher when compared to the other breeds. The slope of linear regressions of ER on MEI indicated that the efficiency of ME use for gain was not different for different breeds. Mean values were 0.58 for

Brangus, 0.61 for Angus and 0.62 for Charolais and were not different. Estimates of maintenance (ME_m) of 0.49 $MJkg^{-0.75}d^{-1}$ for Angus and Brangus and 0.52 for Charolais were also not significantly different.

4.3.3 Metabolic Response to Feeding

In the three breeds, heat production was consistently higher ($P < 0.05$) at the 2.2M intake compared to the 1.2M intake. The parameters related to efficiency measured were not markedly different between breed types, though the Charolais cattle had a tendency for a lower percent change in energy expenditure in response to feeding compared to the other breeds. Therefore the charolais' adaptive response to feeding yields a result that is consistent with the slightly higher efficiency of ME utilization above maintenance for this breed (described further in Moibi et. al., to be submitted).

4.3.4 Variation in Heat Production Amoung Individual Animals Within a Breed.

There was larger variation in heat production among individuals within a breed than between the different breeds. The coefficient of variation for HP among animals within each breed was 24.6%, 24.9% and 16.7% for Angus, Brangus and Charolais groups, respectively.

The absolute heat production data was collected for all animals using indirect respiration calorimetry, and a complete review of this data is included in a separate publication (Moibi et. al., unpublished). The heat production values were utilized in this study as a comparison to our mRNA gene expression analyses. In addition, metabolic parameters such as digestibilities, metabolizable energy intake (MEI), metabolizable energy of maintenance (MEm), energy retention (MER) and average daily gain (ADG) were also collected and utilized as comparison to mRNA expression data contained in this experiment. Independent reporting and analyses of these metabolic data are contained in two separate publications (Moibi et. al., unpublished , Li et. al., unpublished).

4.3.5 Gene mRNA Expression Data

Initial screening of our bovine tissues for neuropeptide Y (NPY) mRNA expression revealed very low if any expression of this gene in our selected tissues. In fact our criteria of 40 PCR cycles failed to amplify any detectable NPY specific signal. We validated the effectiveness of our NPY specific primers by demonstrating positive signals, through RT-PCR analyses, in Bovine hypothalamic total RNA (results not shown). Since hypothalamic tissue is the primary source of NPY, we conclude that the absence of any significant detection of NPY mRNA in muscle, adipose tissue and visceral tissues can be interpreted as an accurate assessment of very low, if any NPY expression in these tissues.

Since appreciable mRNA for NPY appears to be absent in the selected tissues outside the hypothalamus, the tissues that were examined and found to express NPY specific receptor message must be under the control of circulating NPY.

However, the RT-PCR analyses of mRNA for leptin and leptin receptor and NPY receptor in the selected bovine tissues has demonstrated wide spread expression of these genes (examples in Figure 4-1, 4-2, 4-3). Leptin mRNA expression was observed in the three adipose depots examined in this study; mesenteric, peri-renal and subcutaneous, as well as the *biceps femoris* skeletal muscle. Although leptin has been reported in the stomach of humans and mice (Bado et al, 1998), the current study failed to observe any leptin mRNA expression in the corresponding abomasum of the steers, even after as many as 40 PCR cycles. The expression of leptin receptor mRNA was more broadly dispersed across the selected tissues, and was present in total RNA extracted from skeletal muscle, cardiac muscle, rumen papillae, abomasum scrapings, duodenal scrapings, liver, mesenteric adipose, subcutaneous adipose and peri-renal adipose (Tables 4-1, 4-2 & 4-3). In contrast the NPY receptor mRNA was detectable in the three adipose depots, skeletal and cardiac muscle, and duodenum, liver and rumen (Tables 4-1, 4-2 & 4-3), but absent at our stringency of 40 maximum PCR cycles in the abomasum.

4.3.6 Breed and Feeding Level

The statistical evaluation of the mRNA expression of genes and their relationship to feeding levels and breeds identified a few significant differences attributable to such groupings. Although there was substantial variability between individual animals in cardiac muscle leptin receptor mRNA, there was a modestly significant higher level of this message observed in Brangus (Table 4-1) than the other breeds (p=0.07). Another breed specific difference related to the NPY receptor mRNA expression observed in the subcutaneous adipose where the Charolais had a slightly higher average expression of this message than either of the other two breeds examined (Table 4-3) (p=0.08). It should be noted that our subjective assessment was that the Charolais were the most lean animals in terms of subcutaneous adipose, thus the observed increase in NPY receptor mRNA, in this tissue, may be indicative of a different metabolic state, degree of maturation or reduced exposure to nutrients particularly in terms of lipid for storage. Since elevated expression of NPY receptor would result in an increased tissue sensitivity to circulating NPY, which is known to encourage energy conservation and nutrient storage perhaps the subcutaneous adipose tissue in the Charolais are poised to store more lipid if it were to become available, maybe more so than animals that already have higher levels of stored triglycerides. This supposition may be supported by the fact that there was also a breed*feeding level interaction that was significant and indicated a trend where the highest NPY receptor mRNA was present in the Charolais with the lower plane of nutrition (Charolais 178.3 {1.2X average} vs.149.8 {2.2 X average}, p=0.08). The leptin mRNA expression in adipose tissue also seems to be influenced by breed with the average leptin mRNA present in mesenteric adipose in Angus, Brangus and Charolais being 476.6± 8.5, 476.7± 8.1 and 499.4±8.1, respectively. It appears that is a trend for Charolais to express a slightly higher amount of leptin message in their mesenteric adipose (p=0.09). In slight contrast to this observation, the respective expressions of leptin mRNA in peri-renal adipose indicate that Angus have reduced expression (p=0.02) as compared to Brangus and Charolais with average±SEM expressions of 425.7 ± 8.8, 461.1 ± 8.3, 452.2 ± 8.3, respectively. There also appeared to be a significantly different breed*feeding level response in terms of leptin mRNA expression in mesenteric adipose with the Angus and Brangus showing slight increases in leptin expression in 2.2X maintenance versus 1.2X maintenance planes of nutrition, which is what would be expected considering leptin's established role in nutrient sensing and satiety. However, the Charolais breed had reduced mesenteric adipose leptin mRNA in 2.2X versus 1.2X planes of nutrition (p=0.03).

The expression patterns of these genes varied so tremendously between individual animals (Tables 4-1, 4-2 & 4-3) in all the tissues types screened that it is possible that some differences between breed or treatment groups were masked by the differences between individuals within all breeds and feeding levels. It was somewhat of a surprise that comparison of the feeding levels with mRNA expression only detected a single significant difference, solely attributable to nutritional plane, in the expression of these genes given their relationship to satiety, intake and cellular metabolism. The liver NPY receptor expression average ± SEM for the 2.2X maintenance vs 1.2X maintenance planes of nutrition were moderately different (p=0.09) with respective values of 379.8 ± 7.9 and 360.2 ± 7.7. It is a possibility that the extent of our modest difference in feeding level, 1.2X maintenance versus 2.2X maintenance, was insufficient to induce more substantial and consistent variation in the gene expression profiles of these candidate genes especially considering the large individual animal variability that was observed in the mRNA expressions.

It is also possible that had the protein levels been measured there may have been significant differences, and that different sampling times may have elucidated additional variation in the mRNA expressions. Regardless, given the fact that the broad spectrum screening of these genes in bovine tissues was the first of its kind the mere presence and detection of message within these bovine tissues can be considered important. Of course identification of tissue specific mRNA is but the first step towards understanding the significance of these genes in cattle and a foundation towards better understanding the physiological significance of their individual distributions.

Although the current study has detected message for both leptin receptor and NPY receptor in cardiac muscle, liver, rumen papillae and duodenum, it has further been determined that leptin message is notably absent in all of these tissues. This excludes appreciable autocrine effects of these peptides but the receptor presence suggests existence of endocrine or paracrine regulation of these tissues by circulating ligands. The screening of abomasum failed to detect any appreciable leptin mRNA or NPY receptor message, but did reveal that leptin receptor mRNA is expressed. The absence of leptin transcript in bovine abomasum mentioned previously was somewhat surprising since it has been shown to be present in the fundus of both rat and human stomachs. Perhaps this serves as further emphasis of the differences in the digestive tracts of ruminants and monogastrics, and emphasizes the need to perform bovine specific research. The existence of message for leptin receptor in the abomasum infers that circulating leptin from other endogenous sources potentially influence the physiological status of this tissue. This may be related to post-prandial satiety signaling given that leptin itself is often referred to as the satiety hormone but is also known that leptin is involved in the partitioning of nutrients (Scarpace et al, 1998 and Rouru et al, 1999).

Observation of specific mRNA message, for leptin and its receptor, within the three adipose depots and skeletal muscle, could represent an autocrine role within these tissues, in addition to the systemic endocrine lipostatic function that is well documented for leptin. It is known that leptin release from adipose tissue is mediated by nutrient availability associated with satiety as well as accumulation of stored fatty acids. Further examination of the expression and release of leptin in both adipose and skeletal muscle will be required in order to ascertain the complete and specific role of this peptide hormone in bovine.

The presence of leptin receptors in various regions of the bovine digestive tract begs the question as to the physiological relevance of such a distribution, but does evoke plausible hypotheses relating to the possibility that leptin may mediate the manner by which these digestive tract regions process and partition post-prandial substrates. The distribution of both receptors in liver and cardiac muscle extend the concept that serum leptin and NPY levels may be important mediators of whole animal metabolism in cattle, and further individual animal variability may in the future help to explain more of the cause for variability between animals offered the same nutritional plane and composition.

The analyses of mRNA expression levels for the Leptin receptor gene and the NPY receptor gene in cattle *Biceps femoris* tissue has demonstrated that these two genes are expressed in individual animals in a wide range of levels. Moreover, the expression of these genes or, regulation of their respective expressions, are highly linked given their correlation of r = 0.91 in skeletal muscle of 35 steers (Figure 4-4 and Figure 4-5, Murdoch et. al., 2000).

4.3.7 Comparison of Tissue Specific mRNA Expression and Metabolic Data

Analyses of the tissue specific gene mRNA expression relative to measured heat production values failed to identify any strong correlations. There was a modest indication of a possible trend that the NPY receptor mRNA expression in the liver may be positively correlated with heat production but this didn't reach statistical significance (r = 0.26, p=0.13). Interestingly, however when the NPY receptor mRNA present in the liver was compared to MER there was a significant positive correlation (r = 0.35, p=0.05). Since the liver receives early exposure to absorbed nutrients, perhaps the relative expression of NPY receptor mRNA in this organ may reflect some indices of efficient adaptation to abundant nutrients in animals that would correspondingly display improved energy retention.

The average daily gain in the animals was significantly correlated to the leptin receptor mRNA determined in mesenteric adipose (r = 0.43, p=0.02) and also a trend for the same in subcutaneous adipose (r = 0.30, p=0.10). Moreover upon multiple regression analyses of the systemic leptin receptor mRNA expression in cardiac muscle, liver, rumen, mesenteric, peri-renal and subcutaneous adipose there was again a significant association with the measured ADG (r = 0.41, p=0.03).

The extrapolation of ME of maintenance for each animal did not reveal any significant breed differences. The NPY receptor mRNA expression in cardiac muscle trended towards a negative correlation (r = -0.33, p=0.07) with this maintenance requirement, whereas ME for maintenance was positively correlated to NPY receptor mRNA found in subcutaneous adipose tissue (r = 0.38, p=0.04). The leptin receptor mRNA determined in the subcutaneous adipose tissue was also significantly correlated (r = 0.32, p=0.08) to ME maintenance and regression of systemic leptin receptor mRNA expression across tissues, as described earlier, trended towards significance (r = 0.30, p=0.10).

Our examination of leptin mRNA expression in mesenteric adipose tissue displayed a similar trend with ME maintenance (r = 0.30, p=0.10) and was positively correlated with metabolic body weight (r = 0.44, p=0.01). Body weight was further positively correlated to leptin mRNA abundance in subcutaneous adipose tissue (r = 0.41, p=0.02).

The efficiency of energy retention above maintenance was extrapolated by the slope of the MER/ MEI determined at the two levels of feeding. This DMER/DMEI was negatively correlated to the leptin mRNA present in the peri-renal adipose tissue (r = -0.35, p=0.05) and positively correlated with the NPY receptor mRNA in cardiac muscle (r = 0.41, p=0.02).

The results of this study offer some interesting relationships to consider between various tissue specific mRNA expression of these selected genes and some of the commonly measured metabolic parameters in beef cattle research.

4.4 Discussion

Although excessive adiposity is generally not a problem, since the downgrading of a carcass due to excessive fat content is a rare occurrence in typical cattle production systems, lean body growth rate, nutrient intake, nutrient partitioning, and energetic efficiency are physiological factors that can influence production efficiency and as such are of interest to animal scientists. Supported by industry observations of variability in these physiological factors between individual cattle that were housed similarly, offered similar diet, and were phenotypically healthy is the concept that different genetics or gene expression patterns are responsible for these differences in cattle. Leptin and neuropeptide Y (NPY) are two peptides that have well-documented and opposing influence on appetite or nutrient intake. Leptin is known to induce satiety, that is reduce nutrient intake, whereas NPY stimulates food intake through appetite induction. The exogenous administration of leptin to animals results in a dose-dependent reduction in food intake, body weight, and fat depots with an increase in energy metabolism (Campfield et. al., 1995; Pelleymounter et. al., 1995; Chen et. al.; 1996; Levin et. al., 1996). It has been shown that the central hypothalamic pathways involved in food intake are influenced by an associated and linked intrinsic system involving both leptin and NPY, as well as some other orectic and anorectic signals (Stephens et. al., 1995; Schwartz et. al., 1996, Lawrence et. al., 1999). It has also been suggested that these two peptides are also involved in the secretion of growth hormone which is extremely important for lean body growth and the associated accretion of protein (Vuagnat et. al., 1998, Carro et. al., 1998). Of course the specific cellular effects of these peptides are mediated by their selective cell surface receptor expressions which have been shown to be diversely expressed across tissues in several animal species including rat and sheep (Lollman et. al., 1997, Goumain et. al., 1998, Dyer et. al., 1997), but not well studied in cattle. We hypothesized that the endogenous expression of mRNA specific for these peptides, and their respective specific receptors, may be related to some of the observed physiological and phenotypic differences in cattle. Initial screening of the bovine tissues determined that NPY mRNA was not significantly expressed except in hypothalamic brain tissue (results not shown), thus the context of NPY review in this study was limited to peripheral mRNA expression of its receptor.

The current study confirmed that mRNA specific for leptin is present in bovine sub-cutaneous, mesenteric and peri-renal adipose depots, and skeletal muscle, and further was either insignificantly present or absent in the other screened tissues including the abomasum, despite being present in rat and human stomach (Bado et. al., 1998, Sobhani et. al., 2000).

Based on this observed distribution it seems likely that leptin in bovine is involved in nutrient sensing as in other species and may not only serve a lipostatic role but could also be important to the metabolism within skeletal muscle. Further study of the physiological role of leptin in cattle is essential, especially as there appears to be differences between the ruminant and monogastrics, on which most research is performed, in terms of specific tissue expression profiles.

This study involving examination of breeds, selected due to their expected phenotypic differences, was forecast to have a reasonable probability of elucidating potential breed differences in tissue specific expression of these genes. As illustrated by the results in Tables 4-1, 4-2 and 4-3, the variability in the expression of the mRNA between individual animals regardless of whether from the same breed or different breed was large and may have confounded the ability to clearly discern all pertinent breed and treatment differences. Therefore one should not exclude the possibility that there are other important breed differences in the mRNA expression of these genes that may influence the aforementioned physiological parameters, but rather emphasize that the varied amounts observed in individual animals is substantial within each breed.

The presence of mRNA specific for leptin receptor has been demonstrated before in other species (Lollman et. al., 1997), but not in cattle. There has not been much selective research pertaining to the specific function that leptin receptors mediate in cardiac muscle, but this study indicates that there was a trend for increase in this mRNA in Brangus relative to the other two breeds ($p=0.07$). It has been shown that genetic mutation of the leptin receptor, such that truncated and leptin insensitive versions are encoded, results in obesity in db/db mice (Tartaglia et. al., 1995, Chen et. al., 1996) and fa/fa rats (Phillips et. al., 1996). Leptin has further been shown to elevate uncoupling protein expression and increase energy expenditure (Scarpace et. al., 1997). An *in vitro* study on 3T3-L1 fibroblasts has provided evidence that leptin may also suppress the activity of the Na^+, K^+-pump which could be relevant in peripheral tissues (Sweeney et. al., 2000). The potential effects of such similar regulation in cardiac muscle in cattle, and the indication that there are breed differences necessitate further research regarding this observation and its physiological implication. If the increase in leptin receptor mRNA is associated with an increase in cardiac muscle leptin receptor then this may indicate increased leptin sensitivity in the Brangus cattle.

Elevated response to leptin may comparably increase energy expenditure in that tissue. Moreover, the possible regulation of the sodium potassium pump could actually influence the myocardial contractile function through influence on the membrane potential as well as repolarization rate.

The detection of a trend for increased NPY receptor mRNA in subcutaneous adipose tissue in the Charolais (p=0.08) relative to the other breeds examined is quite interesting in the context of our subjective observation that the Charolais were perhaps the most lean of the breed types. In a review of research relating to the function of NPY, Rohner- Jeanrenaud outlines consistent endogenous increases in NPY during fasting, and promotion of food intake and body weight gain when exogenous NPY is administered (2000). Furthermore, NPY influences systemic metabolism as it increases insulin and corticosteroid release and elevates adipose tissue lipogenic activity (Zarjevski et. al., 1994). Perhaps the elevated NPY receptor mRNA in the lean Charolais cattle reflect a physiological attempt to maximize responsiveness to circulating NPY in hopes of maximizing adipose storage upon nutrient availability. This hypothesis is supported by the fact that the Charolais cattle on the lower plane of nutrition had the highest levels of subcutaneous adipose NPY receptor mRNA. Since deposition of subcutaneous adipose is typically curtailed in favour of lean body growth in young cattle perhaps the elevated NPY receptor mRNA in this tissue may be a better indicator of relative maturity of the subcutaneous adipose depot itself. Since subcutaneous adipose tissue is more accessible for sampling than central adipose depots, it will be interesting and quite feasible to evaluate NPY receptor expression in more detail in both physically immature and mature cattle of several breeds to ascertain whether this corollary is maintained and supported.

The NPY receptor mRNA present in the liver, when averaged across all three breeds of cattle, trended slightly higher (p=0.09) in cattle fed at 2.2X maintenance relative to those fed at 1.2X maintenance. The nutrient exposure in the hepatic tissue is exceptionally high as it receives direct exposure to nutrients absorbed from the GI tract via portal blood supply, and we may speculate that the cattle on the higher plane of nutrition may experience reduced levels of circulating NPY relative to the cattle fed at a lower nutritional plane. There is limited research published regarding hepatic NPY receptor expression and to our knowledge this study represents the first evaluation of this in bovine species. One can only speculate in terms of the significance and function of varied NPY receptor expression in cattle. One may hypothesize that hepatic tissue may respond to a lower circulating NPY level by increasing its NPY receptor mRNA and perhaps this explains our observation. A review regarding central regulation of hepatic function suggests that NPY is important for GI tract preparation and handling of nutrients as it stimulates gastric acid and pepsin secretion in dogs and pancreatic secretions in rats (Yoneda et al, 2001). NPY also promotes bile secretion in rats and dogs (Yoneda et. al., 1995). If NPY acts in a similar fashion in cattle then the relative expression of the NPY receptor would correspond with the potential for NPY to prepare the hepatic tissue for nutrient exposure and handling. It may be essential for cattle on the higher plane of nutrition to respond more rapidly in preparation for food intake so as to handle the exposure to increased nutrients and this may partially explain the observed increase in NPY receptor mRNA in the steers on higher nutritional plane as compared to those on the lower feeding level.

In contrast to NPY and its receptor, much more research has been directed at the examination of leptin and its receptor, some of which has been performed in ruminants. Not the least of this research included recent studies indicating that leptin is associated with carcass composition (Buchanan et. al., 2002, Geary et. al., 2003) and that a decrease in adipose leptin mRNA expression in sheep is associated with lactation induced hyperphagia (Sorensen et. al., 2002). These recent findings support earlier reports linking leptin mRNA to backfat thickness (Robert et. al., 1998) and decreased leptin mRNA expression in white adipose of feed restricted animals (Scarpace et. al., 1998). A study by Delavaud et. al., (2002), examined plasma leptin concentration in adult cattle of different breed, adiposity and feeding level and found that plasma leptin was positively correlated with fat mass, but there were no apparent breed differences between fat Charolais and fat Herefords. Plasma leptin appeared to be positively regulated by energy intake. This agrees with Amstalden et. al., (2000) who reported that acute feed restriction decreased both adipose tissue leptin mRNA and plasma leptin levels in heifers. It is important to mention that in lean animals restricted intake did not significantly influence plasma leptin (Delavaud et. al., 2002). This latter point may explain why our modest feed restriction in growing, and reasonably lean steers did not parlay into significant differences in our tissue specific leptin mRNA levels, including those in the three adipose depots that were analyzed.

This current study with young and growing steers did identify modest breed differences in the leptin mRNA expression in mesenteric adipose (p=0.09), which is in contrast with what Delavaud et. al. (2002) reported for fat cattle. Secondly, Delavaud's study compared Charolais and Hereford, two relatively large phenotypes while our comparison was perhaps more extreme as we compared Charolais with Angus and Brangus. Finally, it is possible that the observed significant breed difference in mesenteric adipose leptin mRNA levels may not have resulted in significantly varied plasma leptin levels, with the opposite being true with Delavauds study. One further breed effect with regards to leptin mRNA expression was noted in the screening of peri-renal adipose where there was a reduced expression level in the Angus steers relative to the other breeds (p=0.006 vs Brangus, p=0.04 vs Charolais). It is quite possible that the observed breed differences in various adipose depots leptin mRNA expression may represent different breed specific nutrient partitioning and adipose cell size and or lipid content though we do not have the data to confirm this suggestion.

One surprising observation relating to the leptin mRNA expression in mesenteric adipose was associated with the counter intuitive interaction between breed and feeding level. The Angus and Brangus breeds showed increases in leptin mRNA in mesenteric adipose of steer at the higher feeding level relative to lower feeding level but in Charolais the relationship was reverse. It appears that reduced leptin mRNA was present in mesenteric adipose of the higher feeding level Charolais than those on the lower nutritional plane (p=0.03). It is possible that this observation relates to the observation by Delavaud et. al., (2002) that found that lean animals did not have lower plasma leptin levels upon feed restriction, though feed restricted fat animals did show reduced plasma leptin. Since the Charolais were subjectively ascertained as lean perhaps their counter intuitive expression levels in mesenteric adipose are related somehow to an auxiliary regulatory mechanism of leptin gene in immature, growing and lean cattle.

Once again, in the context of the feeding level treatments, this studies ability to detect significant differences in mRNA expression may have been masked by the dramatic individual animal variability within as well as between different planes of nutrition. Ahima et. al., (1996) demonstrated that serum leptin decreased significantly in fasted mice. In contrast, leptin gene expression is increased in both muscle and adipose as part of a nutrient-sensing pathway responsive to hyperglycemia, hyperlipidemia and glucosamine (Wang et. al., 1998). Li et. al., (1998) showed the increase in hypothalamic NPY mRNA was accompanied by leptin gene suppression in white adipose in fasted rats. The receptor for NPY is also up-regulated by fasting (Zammaretti et. al., 2001), while the leptin receptor is also influenced by diet (Heshka and Jones, 2001).

Since the vast majority of studies have utilized extreme fasting and *ad libitum* planes of nutrition it is possible that our very modest lower plane of nutrition (1.2X) was insufficient to observe dramatic influence on the gene expression profiles as compared to our full-fed steers (2.2X). Furthermore, given our limited sampling times it is also possible that we may have missed some acute effects that our plane of nutrition may have had on the relative mRNA expressions. It was actually part of the experimental protocol to allow sufficient acclimation to the planes of nutrition prior to tissue sampling and as such observe indications of gene expression adaptations that were more chronic in nature.

If we accept that the NPY receptor mRNA, leptin receptor mRNA and leptin mRNA expression profiles are proportional to their specific protein level and that they are similarly associated with appetite, nutrient sensing, and cellular metabolic responses in cattle as they are in other species examined, then the observed variability is pertinent to livestock production. The observed variability in these gene expression profiles might reflect some substantial variation between these individual animals' metabolic status, in spite of offering the same diet and environment and only a modest difference (both above maintenance) in the nutritional plane. Moreover, these results suggest that there may be some physiologically significant consequences that are related to the tissue specific mRNA expression profiles that relate to some of the known production variation of beef cattle and subsequent research directed at elucidating these is necessary.

This study provided an opportunity to compare tissue specific mRNA expression of several genes that were plausible candidates for influencing animal metabolism and physiology with some of the typical metabolic data collected in modern cattle research experiments. Although some interesting correlations were identified between the relative mRNA abundance of the candidate genes in tissues and indices of energy metabolism it is imperative that one remembers that correlations do not establish cause nor do they establish physiological effect. We should consider significant correlations important as they assist in the ability to assess associations between reasonably compared parameters.

The liver expression of NPY receptor mRNA was positively correlated to MER (p=0.05). Yoneda et. al. (1995, 2001) suggested the hypothesis that NPY is important for preparation of GI tissues for nutrient handling, that includes induction of various GI secretions. Therefore steers displaying higher NPY receptor mRNA in their hepatic tissue may process and handle nutrient exposure better than steers less responsive to NPY, thus rendering them more efficient in terms of systemic energy retention. The significance of net nutrient metabolism by the liver was shown to be an important component of energy metabolism in growing beef steers (Reynolds et. al., 1992), and supports the premise that hepatic nutrient handling could significantly impact the MER measured in growing steers.

Furthermore, increased message for NPY receptor may reduce the hepatic energy expenditure by facilitating NPY induced reduction in lipolysis, glycolysis and oxygen consumption. Given the typically high metabolic rate in the liver as well as its considerable size a small increase in hepatic efficiency this may translate into a noticeable increase in systemic efficiency and thus systemic energy retention given all other things are equal.

Although, average daily gain doesn't necessarily indicate absolute energy storage as it fails to differentiate between lean body growth and fat deposition, in our young growing cattle that are only accumulating modest adipose stores it still represents an easily obtainable index of growth. We found that the leptin receptor mRNA expression in both mesenteric adipose (p=0.02) and subcutaneous adipose were positively correlated with ADG. Moreover multiple regression analyses of the leptin receptor mRNA expression in cardiac muscle, rumen and the three adipose depots showed a strong positive correlation with ADG (r=0.41, p=0.03). Therefore, the systemic mRNA expression of leptin receptor mRNA in multiple tissues in an individual animal is representative to some degree of that animal's propensity for growth as indicated by ADG measurement. This may seem counterintuitive given that the effect of leptin would be expected to reduce nutrient retention, but this study may have indirectly differentiated between animals that are more predominantly undergoing lean body accretion rather than adipose accretion. That is animals that may have been storing more fat would be associated with reduced ADG when compared to another animal that stores the same amount of ME as muscle, due to the differences in energetic density and water retention between these two tissue. In the steer with decreased lipid storage there may be a decrease in leptin, which may parlay into an increase in tissue leptin receptor expression if there is a ligand-receptor feedback pathway in these tissues.

The energy required for maintenance (MEm) is important to the objectives of the livestock producers if we consider that the lower this energy cost, the greater proportion of ME can be utilized for growth, that is tissue deposition. When the MEm is extrapolated for each steer and compared with tissue specific mRNA measurements this study revealled some interesting relationships. There appears to be a divergent relationship between the NPY receptor mRNA expressed in the cardiac muscle and subcutaneous adipose as there was a negative correlation between cardiac muscle NPY receptor mRNA (r = -0.33, p=0.07) and MEm whereas there was a positive relationship with this maintenance cost and NPY receptor mRNA in subcutaneous adipose (r = 0.38, p=0.04). This emphasizes the necessity to evaluate differently the physiology behind the receptor profiles in essential core organ tissues as compared to tissues of predominantly nutrient storage. It is not surprising that these two different tissues may have opposing relationships to systemic metabolic parameters especially when related to nutrient intake and perhaps core sparing versus nutrient depot homeorrhetic physiology. The mRNA for leptin receptor was also positively correlated with individual animal maintenance costs in subcutaneous adipose (r = 0.32, p=0.08), which suggests that the set point of this adipose depot's response to the nutrient sensing pathway involving both leptin and NPY may be coordinately regulated and this regulation is linked to conditions supporting, evoking or resulting from higher maintenance costs. Furthermore, the leptin mRNA present in mesenteric adipose was also related to MEm (r = 0.30, p=0.10) and metabolic body weight (r = 0.44, p=0.01). The leptin mRNA in subcutaneous adipose was also strongly correlated with the metabolic body weight(r = 0.41, p=0.02). Thus the larger animals with perhaps more lipid stores not surprisingly express increased leptin mRNA in their most abundant adipose depots, mesenteric and subcutaneous.

The DMER/DMEI represents the efficiency of energy retention for our steers fed at above maintenance requirements (between 1.2X and 2.2X maintenance). The greater the value the more efficient these animals convert MEI into retained energy as body mass accretion or growth. The leptin mRNA expression in peri-renal adipose appears to be negatively correlated with this measure of anabolic efficiency (r = -0.35, p=0.05), where as NPY receptor mRNA expressed in cardiac muscle is positively associated (r = 0.41, p=0.02). Although, such correlations are interesting the true value of these relationships will be obtained upon acquisition of the physiological explanation and an understanding the tissue specific regulation of these candidate genes.

This study has made strides towards the tissue specific evaluation of several genes that may have physiological significance to the individual animals metabolic efficiency and may ultimately assist in our understanding of some of the parameters responsible for variability in ruminant growth, nutrient intake and efficiencies of energy retention.

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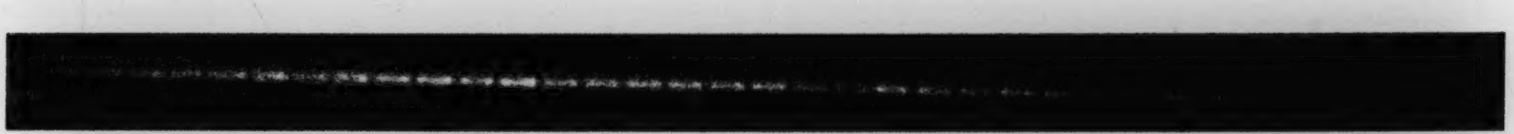

Figure 4-1 Ethidium bromide stained image of separation of Mesenteric adipose leptin mRNA specific amplicon on 2% (w/v) Agarose Gel each lane represents an individual animal. First lane is 100 bp molecular weight marker.

Figure 4-2 Ethidium bromide stained image of separation of leptin receptor mRNA specific amplicon (lower band) with internal control G3PDH amplicon (upper band) on 2% (w/v) Agarose Gel each lane represents an individual animal. First lane is 100 bp molecular weight marker.

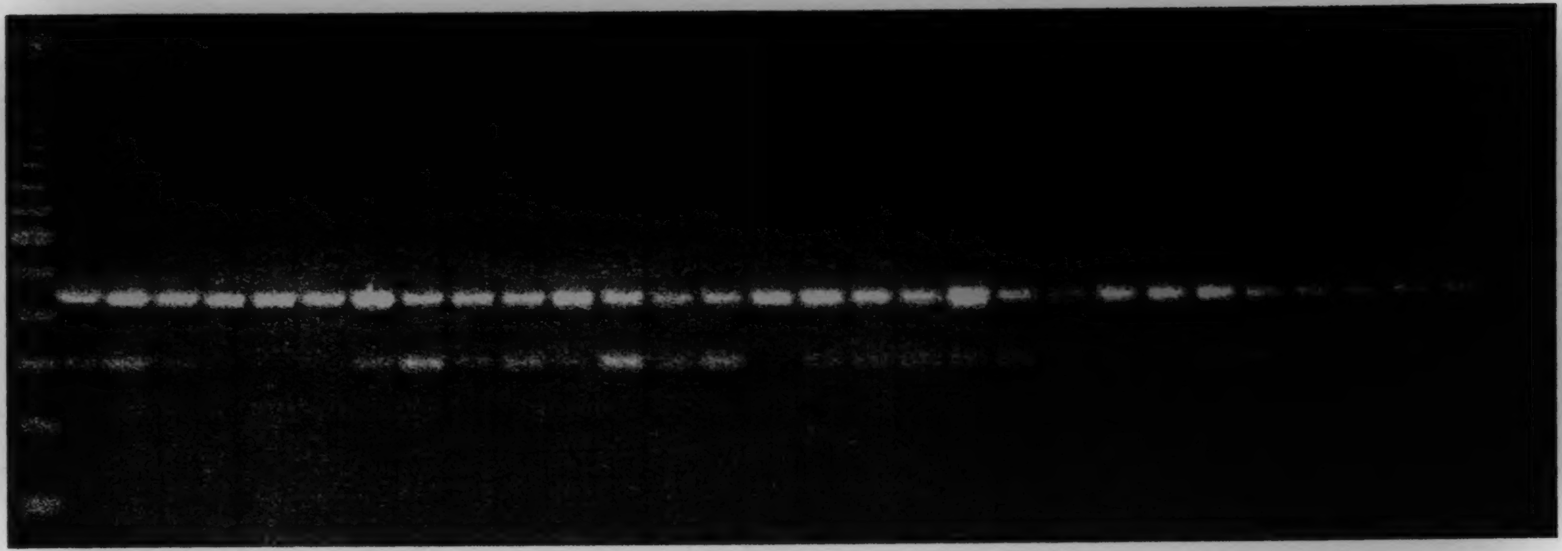

Figure 4-3 Ethidium bromide stained image of separation of NPY receptor mRNA specific amplicon (lower band) with internal control G3PDH amplicon (upper band) on 2% (w/v) Agarose Gel each lane represents an individual animal. First lane is 100 bp molecular weight marker.

Table 4-1 Effect of breed and feeding level on mRNA expression^z of leptin, leptin receptor and NPY receptor in muscle.

	Leptin receptor cardiac muscle	NPY receptor cardiac muscle	Leptin biceps femoris	Leptin receptor biceps femoris	NPY receptor biceps femoris
Treatment					
2.2X	63.69	44.01	45.99	30.40	34.70
SD	34.41	30.53	28.80	23.10	44.40
1.2X	58.04	50.57	46.40	46.10	46.50
SD	26.04	33.92	29.41	41.70	59.00
Breed					
Angus	58.56	50.94	62.19	39.30	41.80
Brahman-Angus	68.10	52.32	33.30	48.10	52.80
Charolais	53.94	39.30	39.08	32.80	33.30
pooled SD	16.05	32.02	28.62	34.70	52.00

[z] arbitrary densitometric units, mean values. SD = standard deviation

Table 4-2 Effect of breed and feeding level on mRNA expression^z of leptin and NPY receptors in liver and GI tissues.

	Leptin receptor rumen	NPY receptor rumen	Leptin receptor duodenum	NPY receptor doudenum	Leptin receptor liver	NPY receptor liver	Leptin receptor abomasum
Treatment							
2.2X	104.28	821.56	448.91	442.59	252.32	379.71	74.06
SD	58.77	64.00	25.29	23.59	66.67	36.79	42.38
1.2X	96.67	833.98	445.02	459.25	244.87	360.18	61.43
SD	61.04	66.14	38.78	50.30	62.21	26.24	53.02
Breed							
Angus	99.08	827.35	458.87	454.22	250.22	369.14	75.87
Brahman-Angus	79.53	830.43	436.20	460.40	248.44	360.37	57.34
Charolais	120.77	826.26	444.77	439.62	246.81	378.71	68.64
pooled SD	59.19	64.45	32.51	39.98	63.57	32.85	47.87

[z] arbitrary densitometric units, mean values. SD = standard deviation

Table 4-3 Effect of breed and feeding level on mRNA expression[z] of leptin and NPY receptors in adipose depots.

	Leptin mesenteric adipose	Leptin receptor mesenteric adipose	NPY receptor mesenteric adipose	Leptin subcutaneous adipose	Leptin receptor subcutaneous adipose	NPY receptor subcutaneous adipose	Leptin perirenal adipose	Leptin receptor perirenal adipose	NPY receptor perirenal adipose
Treatment									
2.2X	484.97	113.80	33.12	684.81	175.55	151.09	455.00	41.29	50.55
SD	26.45	33.60	18.83	62.85	41.98	26.66	41.22	30.50	40.68
1.2X	483.33	101.98	47.63	667.82	169.34	143.45	439.50	41.03	61.98
SD	35.71	20.26	29.67	57.19	61.09	43.92	20.43	27.06	34.25
Breed									
Angus	473.48	102.35	41.92	676.94	151.93	126.84	425.66	38.68	51.76
Brahman-Angus	479.13	108.70	40.73	646.68	187.33	150.94	464.75	42.30	66.15
Charolais	499.35	112.20	39.12	702.15	179.06	164.02	452.16	42.68	52.18
pooled SD	31.11	27.79	25.71	59.73	52.01	36.25	32.71	28.28	37.40

[z] arbitrary densitometric units, mean values. SD = standard deviation

Table 4-4 Correlations of mRNA abundance^{z} with measures of energetic efficiency and heat production

	Leptin receptor cardiac muscle	**NPY receptor cardiac muscle**	**Leptin Biceps femoris**	**Leptin receptor Biceps femoris**	**NPY receptor Biceps femoris**
Metabolic Parameter					
Heat production	-0.02	-0.16	0.01	-0.14	-0.08
ADG (g/kg$^{.75}$)	0.26	-0.12	-0.11	0.04	0.11
MEI (MJ/kgbw$^{.75}$)	0.12	-0.10	-0.09	-0.20	-0.11
MER corr	0.15	-0.10	-0.13	-0.23	-0.12
DMER/DMEI corr	-0.01	**-0.41**	0.07	-0.13	-0.21
ME maintenance	0.00	**-0.33**	0.02	-0.09	-0.01

n = 35. ^{z} arbitrary densitometric units

Table 4-5 Correlations of mRNA abundance^z with measures of energetic efficiency and heat production

	Leptin receptor duodenum	**NPY receptor duodenum**	**Leptin receptor rumen**	**NPY receptor rumen**	**Leptin receptor liver**	**NPY receptor liver**	**Leptin receptor abomasum**
Metabolic Parameter							
Heat production	-0.25	-0.24	0.02	-0.29	0.17	0.26	0.11
ADG (g/kg$^{.75}$)	0.21	-0.05	0.00	-0.05	0.19	0.18	0.11
MEI (MJ/kgbw$^{.75}$)	-0.04	-0.11	0.17	-0.16	0.12	0.18	0.12
MER corr	-0.03	-0.05	0.15	-0.19	0.02	**0.35**	0.08
DMER/DMEI corr	0.10	0.10	-0.12	0.00	0.09	0.08	0.01
ME maintenance	-0.07	-0.13	-0.07	0.16	0.17	-0.08	0.05

n = 35. . ^{z} arbitrary densitometric units

Table 4-6 Correlations of mRNA abundance^z with measures of energetic efficiency and heat production

	Leptin mesenteric adipose	**Leptin receptor mesenteric adipose**	**NPY receptor mesenteric adipose**	**Leptin subcutaneous adipose**	**Leptin receptor subcutaneous adipose**	**NPY receptor subcutaneous adipose**	**Leptin perirenal adipose**	**Leptin receptor perirenal adipose**	**NPY receptor perirenal adipose**
Metabolic Parameter									
Heat production	0.07	0.13	-0.12	0.03	0.15	0.08	0.24	0.07	-0.06
ADG (g/kg$^{.75}$)	-0.28	**0.43**	-0.12	0.01	**0.30**	0.23	0.17	0.07	0.00
MEI (MJ/kgbw$^{.75}$)	0.01	0.26	-0.22	0.14	0.12	0.11	0.04	-0.05	-0.17
MER corr	-0.06	0.25	-0.19	0.10	0.04	0.05	-0.07	-0.15	-0.26
DMER/DMEI corr	0.13	0.18	-0.02	0.02	0.10	0.21	**-0.35**	-0.06	0.06
ME maintenance	**0.30**	0.10	-0.15	0.02	**0.32**	**0.38**	-0.08	0.17	0.22

.n=35 ^{z} arbitrary densitometric units

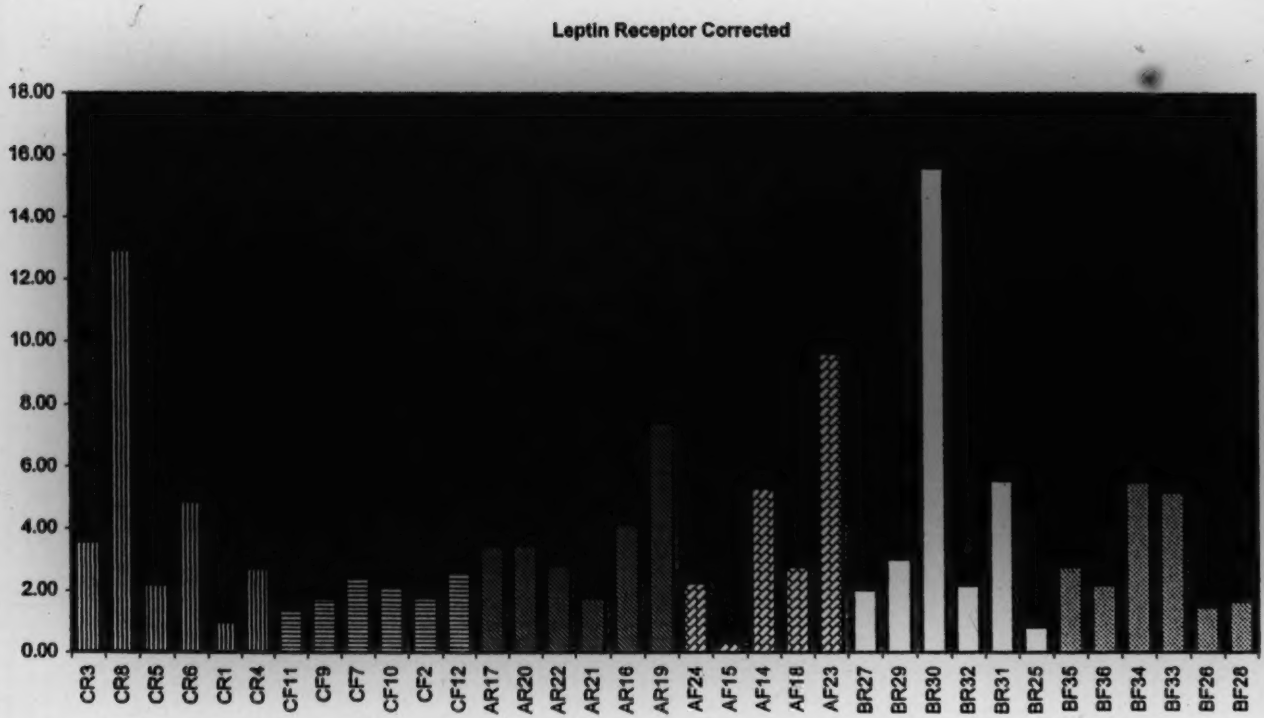

Figure 4-4 Histogram of quantifications of individual animals expression of leptin receptor mRNA^z in *biceps femoris* tissue. Breeds represented are Charolais(C), Angus (A), and Brahman/Angus cross (B). Treatments represented as 2.2X maintenance (F) and 1.2X maintenance(R)

^{z} arbitrary densitometric values

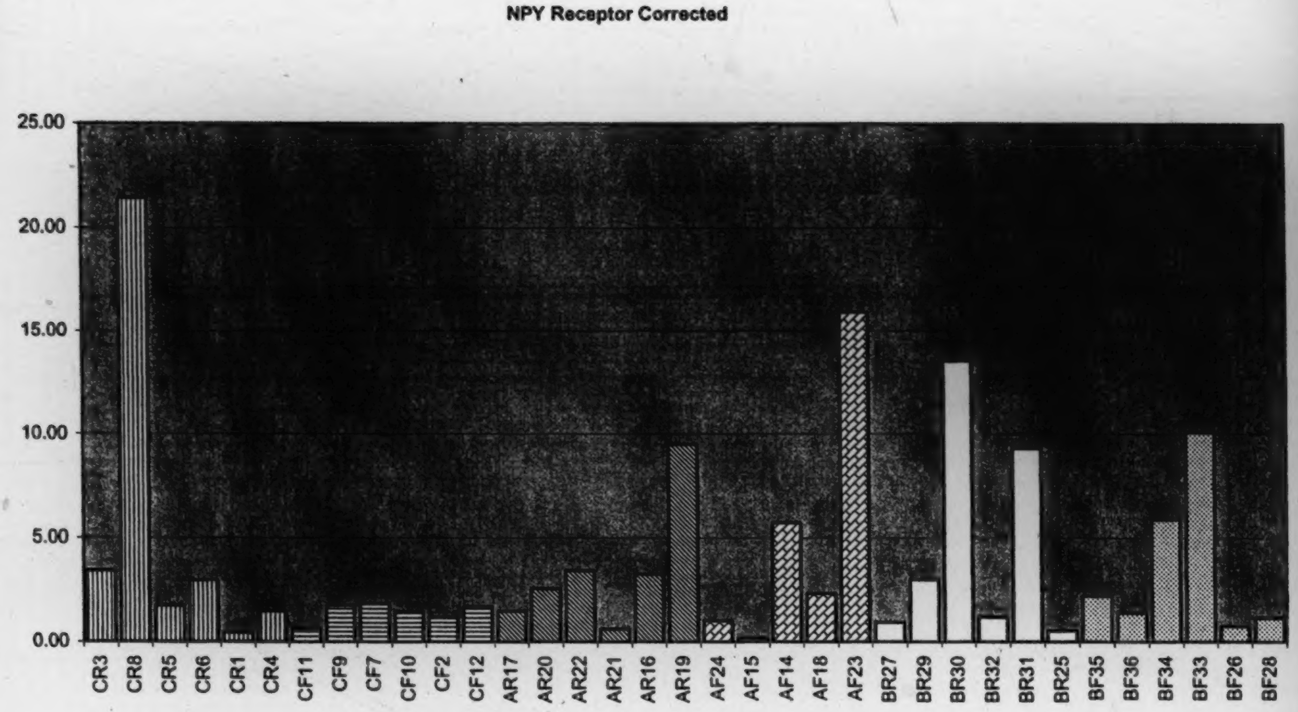

Figure 4-5 Histogram of quantifications of individual animals expression of NPY receptor mRNA^z in *biceps femoris* tissue. Breeds represented are Charolais(C) , Angus (A) and Brahman/Angus cross (B). Treatments represented as 2.2X maintenance (F) and 1.2X maintenance(R)

^{z} arbitrary densitometric values

CHAPTER 5

The Effect of Diet Type, Level, and Environment on UCP-2, UCP-3, Leptin Receptor and Neuropeptide Y Receptor mRNA Abundance in Bovine Skeletal Muscle Biopsies

5.1 Introduction

Although the concept of homeostasis is a sound principal, it is more appropriate to consider livestock as metabolically homeorrhetic given their need to adapt to dramatic environmental changes and physiological states. It is apparent that the ability of cattle to maintain normal internal conditions of body temperature, blood pH and mineral balance under varied nutrient availability, growth rates, age and environmental temperatures requires appropriate physiological adaptation to the given conditions. Some scenarios relevant to livestock production that may affect individual animal efficiency include diet type (roughage vs. concentrate), plane of nutrition (below maintenance, near maintenance, or above maintenance) as well as exposure to cold (or hot) ambient temperatures. Delavaud et.al. (2002), indicated that serum leptin concentrations, measured 4 hours post-prandially, in adult bovine are positively correlated with feeding level, similar to what is observed in studies of other animal species. This seems to confirm an earlier study that reported a fasting induced decrease in obese gene expression in bovine subcutaneous adipose tissue (Tsuchiya et. al., 1998).

Our intent was to characterize the variation in the candidate gene expression in animals exposed to different diet types at two above maintenance feeding levels as well as in the context of indoor and outdoor housing. It was of interest to discern if animals employed similar adaptational strategies, particularly with regard to these candidate gene expression profiles, in response to these conditions or whether some animals were more or less efficient subsequent to adaptation to environmental and dietary treatments. The current study focused on the mRNA abundance of the candidate genes in skeletal muscle, given its importance as the primary marketable product and accessibility for biopsy, and the possibility that the expression profiles may change in this dynamic tissue in the face of some production typical stressors. This study was designed to test the hypotheses that skeletal muscle adaptation to conditions of inside vs. outside, roughage vs. concentrate, and high vs low planes of above maintenance feeding, would be represented by adaptation of mRNA expression of these candidate genes related to energy metabolism.

5.2 Materials and Methods

5.2.1 Animals and Management

Experimental procedures were approved by the Faculty Animal Policy and Welfare Committee as being consistent with the guidelines of the Canadian Council on Animal Care (CCAC 1998)

Thirty eight steers (36 experimental plus two spares) of mixed breeding (BW ± SD, 204 kg ± 20 kg) were brought from the University of Alberta Ranch, Kinsella, Alberta to the Laird McElroy Environment and Metabolism Center located at the Edmonton Research Station, Edmonton, Alberta in early November

1998. Upon arrival the steers were housed outside in pens and were fed a diet consisting of long stem hay (alfalfa/brome grass mix) and rolled barley (50:50 ratio). Prior to the commencement of the study the steers were fed in groups and had constant access to water throughout the adaptation period. Animals were weighed before they were placed on the treatments and at two-week intervals.

5.2.1.1 Animal Health and Training

One week after arrival the animals were identified with ear tags, placed in the animal's right ear. The animals were vaccinated with an 8-way vaccine (Blacklegol 8™, Bayer Corporation, Shawnee Mission, KS, USA), treated for internal and external parasites with pour on invermectin (Ivomec™, Merck AgVet, Kirkland, PQ, Canada), implanted with an anabolic growth promotant (Ralgro™, Scherling Canada Inc., Point-claire, PQ, Canada) and injected with a solution of vitamin ADE. Animals were re-implanted with the same anabolic agent every 90 days.

Animals were halter broken over the next two-four weeks so that animal technicians could restrain and lead the animals during various aspects of the experiment. After sufficient halter training, all animals were accustomed to the metabolic crates and the calorimetry system and procedures.

5.2.1.2 Animal Housing

In early December 1998 animals were placed on feed and adapted to respective experimental diets and feeding levels, as prescribed through random assignment. In late December, 12 animals were moved indoors (20 ± 2°C) and housed in individual pens (2.8 m x 2.8 m). The remaining 24 animals were split into 2 groups of 12 and housed outside (-12.5 ± 3°C for period 1 and –6.7 ± 2°C for period 2) in two pens (10 m x 7.5 m). The outside pens did not have any overhead shelter except for a 2 m overhang that covered the feeding area.

Indoor pens were equipped with feeders for individual animal feeding and watering. Outside pens were equipped with calan gates (American Calan Company, New Hampshire) allowing for individual animal feeding in a group setting. Water for the outside animals, was delivered by heated automatic water bowls (UFA model 50, Calgary, AB, Canada) with sufficient capacity to water 50 mature animals per day.

Wood shavings were used as the bedding material for all animals. Indoor pens were cleaned every Monday and Friday throughout the trial in order to keep the animals clean and dry. Outside pens were bedded with the same wood shavings once a week or whenever weather conditions warranted.

5.2.1.3 Diets

Two diets and two feeding levels were used in this experiment. The composition of the two experimental diets is shown in Table 2.1. The first diet was a roughage-based (R) (75% (w/w) 2nd cut alfalfa/brome grass hay and 25% (w/w) concentrate). The second diet was a concentrate based (C) (based on 90% (w/w) concentrate and 10% (w/w) 2nd cut alfalfa/brome grass hay). The two diets were formulated with locally grown ingredients and were chosen to represent a back-grounding (R) diet and a feedlot (C) diet. These diets were fed at two different levels based on 1.2 and 2.2 times maintenance energy requirements as estimated from the NRC nutrient requirements of beef cattle (NRC, 1996).

Cobalt/iodine supplemented salt and water were offered ad libitum to every animal. The average body weights of the animals were as follow; Period 1 indoor steers 214.21 +/- 20.37 kg, outdoor steers 230.77 +/- 18.45kg; period 2 indoor steers 243.71 +/- 30.57; outdoor steers 247.69 +/- 21.15kg.

5.2.1.4 Energy Balance and Metabolism Measurements

Feed intakes were measured daily from start of adaptation to the end of each experiment period. Heat production and methane (CH_4) production were measured by indirect respiration calorimetry in each period. Fecal excretion of energy and nitrogen were determined by reference to lignin and acid insoluble ash in feed and fecal grab samples collected over a two-day collection during each period. Energy content of feed, feces and urine were determined by Bomb Calorimetry using an adiabatic bomb calorimetry (Leco automatic calorimeter AC 300, St. Joseph, MI, USA).), and nitrogen content of feed feces and urine were determined by Kjeldahl procedure (AOAC).

Energy retention (ER) was calculated as metabolizable energy intake (MEI) minus HP at each feeding level. Metabolizable energy requirement for maintenance (ME_m) was estimated using the determined MEI minus the ER at 1.2M feeding level. Efficiency of energy retention above maintenance was calculated as the ratio of the change in ER divided by the change in ME intake between two feeding levels.

Samples of *biceps femoris* skeletal muscle were sampled via surgical procedures under local anaesthesia (Appendix). These samples were obtained from each animal, following adaptation to, and during, each treatment period. All samples were obtained and immediately frozen in liquid nitrogen to preserve the total RNA until its subsequent extraction.

This approach allowed us to examine the effects of period, environment, and two planes of nutrition on both roughage and concentrate diets, on indices of performance, metabolism and candidate gene expression. Since all of these treatments represent scenarios that a typical production animal may be exposed to, it was essential to discern the effects of these conditions on the expression profiles of our gene candidates in the skeletal muscle.

5.2.2 Sub-components to Experiment

5.2.2.1Diet and Intake Effects:

Indoor animals which received the roughage or concentrate diet during periods one and two were switched to the opposite diet during periods three and four to complete a cross-over design for comparison of roughage vs concentrate at each of two intake levels (1.2M and 2.2M).

5.2.6.2 Environment and Diet Effects:

Effect of environment and diet was determined during periods one and two (January and February of 1999) by comparing data recorded on indoor and outdoor animals during winter. Animals receiving high or low intakes during period one were switched to the opposite level during period two.

5.2.3 Statistical Analyses

Data was analyzed by Analysis of Variance, using the GLM and LSMEANS procedure of SAS. The model included diet feeding level and environment. Comparisons for significant differences made by the probability of differences (PDIFF) option. Relationships between gene expression and indices of energy metabolism were determined by regression analysis.

5.3 Results

5.3.1 Energy Metabolism Parameters

Table 5-1 illustrates the effects of diet type and level of feeding on various parameters of energy metabolism. There were significant effects of diet type on DEI ($P=0.02$), MEI ($P<0.0001$), ER ($P=0.004$), CH_4 ($P<0.0001$). The animals fed the concentrate diet had increased digestible energy intake (by 0.04 MJ/kg$^{0.75}$/d), metabolizable energy intake (by 0.07 MJ/kg$^{0.75}$/d) and, energy retention (by 0.06 MJ/kg$^{0.75}$/d). These differences were quite small when calculated as percentage changes and were a result of small decreases in fecal, and methane energy losses compared to animals fed the roughage based diet.

Diet type had no effect on the HP ($P=0.54$), UL ($P=0.53$), MR ($P=0.78$) but there was a trend ($P=0.07$) for diet type to affect the efficiency of ME use above maintenance. Concentrate diet fed animals tended to be 13% more energetically efficient when fed above their maintenance requirement compared to those on the roughage diet.

As expected, level of feeding had significant effects on the DEI ($P<0.0001$), MEI ($P<0.0001$), ER ($P<0.0001$), HP ($P<0.0001$), and UL ($P=0.04$). Animals fed at the higher level of intake (2.2M) had significantly increased digestible energy intake (by 0.4 MJ/kg$^{0.75}$/d), metabolizable energy intake (by 0.4MJ/kg$^{0.75}$/d), energy retention (by 0.23 MJ/kg$^{0.75}$/d), heat production (by 0.14 MJ/kg$^{0.75}$/d), urinary energy loss (by 0.4 MJ/kg$^{0.75}$/d). Methane loss was increased (by 1.1 MJ/kg$^{0.75}$/d) at the higher feeding level only for the roughage diet

The experimental period had a significant effect on urinary energy loss per day (P=0.02) and a trend (P=0.09) on methane energy loss per day. During period two, animals lost 0.5 MJ/d more energy from urinary sources than during period one, and tended (P=0.09) to lose 0.3 MJ/d more energy from methane sources than during period one.

Table 5-2 shows the effects of environment on the energy metabolism parameters averaged over both periods. On this basis, there were no significant effects of environment on the energy parameters. There were no effects of environment on digestibility or metabolizable energy content of the diet. Although digestibility of roughage-based diets is often reduced in a cold environment, the winter temperatures were not severely cold in this study. On the other-hand, a difference in energy expenditure was expected.

Table 5-3 shows the heat production data further analyzed according to period (also Figure 5-6), to look for longer-term environmental influences. Period one data showed no significant (P=0.60) effect of environment on heat production, but during period two there was a significant (P=0.04) effect of environment on heat production. This indicates that the change in resting heat production with environment required several weeks to develop. After acclimation to the indoor and outdoor environments, the heat production was approximately 10 % higher in steers housed outdoors during winter. This result is similar to results of previous studies, which have shown that cold acclimation increases the energy expenditure of cattle by 10 to 30 % depending upon severity of the weather, age and feeding level, and contributes to a higher maintenance requirement during winter (Figure 5-5, 5-7). Figure 5-1 illustrates that there was a small tendency for efficiency of energy retention to be lower in the outdoor environment, although the difference was not significant

5.3.2 Environment and Heat Production

After acclimation to the indoor and outdoor environments, the heat production was approximately 10 % higher in steers housed outdoors during winter (Figure 5-6). This result is similar to results of previous studies, which have shown that cold acclimation increases the energy expenditure of cattle by 10 to 30 % depending upon severity of the weather, age and feeding level, and contributes to an increase in maintenance requirement (Young, 1975, Young et. al., 1989; Christopherson 1994).

5.3.3 Results of Genetic Analyses

5.3.3.1 Gene Expression Profiles in Muscle Biopsies

The characterization of gene specific mRNA abundance in the skeletal muscle biopsies resulted in several interesting observations (see Figures 5-1, 5-2, 5-3, 5-4). The feeding level alone did not significantly alter the expression of the specific gene mRNA abundance in the skeletal muscle biopsies, which likely reflects the modest degree of difference between the nutritional planes, both of which were above maintenance.

Upon examination of the leptin receptor mRNA abundance and a comparison of treatment mean values, the studies results indicated an interaction (p=0.05) between environment and diet type which reached significance. Whereas the indoor concentrate fed steers showed a trend toward lower leptin receptor mRNA values when compared to indoor roughage fed steers, the reverse was true in the outdoor, housed animals. The outdoor, roughage fed animals showed lower mean leptin receptor mRNA abundance than the outside concentrate fed steers. The mean values for these treatments are shown in table 5-6.

There were no significant differences, attributable solely to diet type, in gene expression in the muscle biopsies from animals fed either roughage or concentrate diets (Table 5-7), that is, diet type alone did not dramatically influence the abundance of the candidate genes mRNA within the *biceps femoris* skeletal muscle. There may be some indication of a weak trend (p=0.155) towards increased UCP-2 mRNA in muscle tissue collected from concentrate fed steers versus roughage fed steers.

Though the current project did not detect any significant changes in skeletal muscle UCP-2 mRNA abundance induced by the environment and diet treatments one should note that there was an observed trend for a period effect suggesting the possibility of decreasing UCP-2 mRNA with age of the animals. It may also be indicative of the fact that upon correlation analyses there was a significant negative correlation (p=0.03) between UCP-2 mRNA abundance and metabolic body weight over the duration of this study. Similarly there was also a negative correlation between NPY receptor mRNA abundance in skeletal muscle biopsies and metabolic body weight (p=0.0004), alluding to the possibility that expression of this mRNA may be inversely associated with animal maturity and/or size.

Additionally, the expression of NPY receptor message tended to be decreased in the outside winter acclimated steers whereas UCP-3 mRNA abundance trended towards an increase ($P<0.10$) in the animals housed outside versus those housed inside during winter

5.4 Discussion

Skeletal muscle tissue is of particular importance in livestock production, not simply because it is the marketable product in the carcass, but also because it represents such a large proportion of the body mass (50-60%). Furthermore, skeletal muscle is extremely metabolically active, in particular in the growing animal, and is known for its adaptive strategies including, but not limited to, such things as shivering and non-shivering thermogenesis. It is imperative that as animal scientists one endeavours to understand the physiological parameters that influence bovine muscle homeorrhesis, mediate its growth and adaptational strategies, and ultimately affect its relative efficiency for protein accretion and nutrient utilization, while maintaining the ability to yield high quality attributes as marketable meat. This study was designed so as to increase the available bovine specific knowledge with regards to skeletal muscle adaptation to practical stressors related to production, in terms of the muscles mRNA expression of candidate genes believed to influence its metabolic state.

The lack of significant skeletal muscle response to diet type may seem surprising since diet type has been shown in other studies, on monogastric animals, to influence uncoupling protein leptin receptor and NPY receptor expression. It should be kept in mind that most studies on other species that have found effects of diet type on mRNA expression profiles have related to variability in the lipid content (Matsuda et. al., 1997, Surwit et. al., 1998) and or total energy content. Perhaps the fixed feeding levels used in this study provided too narrow a range of lipid content and energy (both above maintenance requirements) to influence muscle expression of the particular genes that were examined. Moreover, it is not without precedent that nutritional effects measured in monogastric species are determined to be less than comparable when measured in ruminants. It is also feasible that the diet type may slightly influence the expression of our candidate gene mRNA but that individual variability is such that the relatively small trends were masked.

This study did reveal some effects of diet type in terms of it's interaction with environment and it's influence on leptin receptor mRNA abundance. When one considers the interaction of diet type and environment with respect to leptin receptor mRNA abundance in the skeletal muscle biopsies it points to the need to consider the complexity of sensory and regulatory processes that mediate metabolically active tissues such as skeletal muscle. Why might the leptin receptor mRNA be lower in indoor concentrate fed animals versus indoor roughage fed, and show an opposite trend when measured in outside housed steers? Since this study incorporated a single time of feed provision (morning), it is possible that the indoor and outdoor animals may have consumed their respective daily feed at different rates that was dictated according to their metabolic status and hunger. This could result in variability in the post-prandial serum nutrient flux both in terms of extent and timeline. Furthermore, the results showed that the heat production of the outside group was increased by about 10 % over inside housed steers, likely as a result of long-term cold acclimation (in period 2, Figure 5-6). Therefore the differences in the metabolic rates of these two groups may affect and reflect corresponding differences in nutrient acquisition, processing, partitioning and especially utilization.

Since leptin is a component of a nutrient sensing pathway one may expect that animals on the same plane of nutrition, but with different energy status due to cold exposure, would have differing serum leptin profiles such as has been shown (Tsuchiya et. al., 1998, Delavaud et. al., 2002). In an attempt to conserve energy the outdoor animals may reduce their respective leptin receptor mRNA so as to proportionally decrease their skeletal muscle sensitivity to serum leptin. This may be especially apparent in roughage fed outdoor animals where the MEI and ER were significantly lower and thus the need for energy conservation is perhaps the greatest. These of course are simply postulates that might explain this studies results, but further experiments and measurements are required to ascertain their relative validity.

The outside environment, representing longer-term cold acclimation, treatment was associated with a reduction of the mean NPY receptor mRNA in the muscle biopsies (628.27±14.55), when compared to the mean NPY receptor mRNA in muscle from the inside steers (700.88±10.11). Furthermore, the direct negative correlation identified between NPY receptor abundance and heat production measurements in periods one and two (-0.34 and –0.40 respectively) re-enforce and support the expected adaptational response of NPY receptor mRNA towards the need for increased thermogenic capacity in outside animals skeletal muscle. The modest negative correlation that was observed between NPY receptor expression in muscle and heat production may be an example of an association that becomes useful in terms of working towards a marker-assisted selection. Of course this would occur only after further investigation establishes a more refined understanding of this association under more varied conditions of measurement. As this research project has developed the necessary resources for future evaluation of these various candidate genes using molecular biology we are well-positioned to capitalize on the areas where future research is predicated.

The results of this study also established evidence that bovine skeletal muscle may increase it's UCP-3 mRNA content in response to chronic cold acclimation as is apparent upon a comparison of outside mean versus inside mean UCP-3 mRNA abundance (577.62±5.17 and 562.63±3.65, respectively). Up-regulation of skeletal muscle UCP-3 mRNA in this manner may be specifically related to increased thermogenic capacity, through capitalizing on the dissipation of metabolic heat associated with reduced coupling of oxidative phosphorylation to ATP synthesis. Similar observations of increased uncoupling protein expression have been shown in other species of animals in response to cold (Rehnmark et. al., 1992, Larkin et. al., 1997), though rodent skeletal muscle UCP-

3 expression was not associated with environmental temperature in a study by Boss et. al. (1998). The difference in this current study's results may be associated with the differences in species, but also might reflect or be a result of the modest cold treatment (6 °C) and duration (48hours) employed by Boss.

It is important to realize that metabolic adaptation occurred in the steers even though the winter conditions were not particularly severe during the study, with ambient temperatures of the outside group only being modestly cold at a minimum of about -15°C. It is possible that the chronic adaptation of the skeletal muscle UCP-3 mRNA content may have been even more dramatic than what this study observed had the steers been exposed during a winter with lower ambient temperatures. This study's cattle were likely responding to the prolonged (several weeks) of cold exposure and it appears that the cold acclimation in the outside animals may have significantly influenced the expression of the UCP-3 gene in peripheral skeletal muscle. These responses could be an essential component of the adaptive change that ultimately contributes to the ability of cattle to thrive in a cold environment. This study's results show that the animals housed outdoors had significantly increased rates of heat production (10 % higher) compared to those in a warmer indoor environment with an association to UCP3 mRNA, in skeletal muscle, the only tissue that could be easily biopsied during this study. It would clearly be of importance to extend these studies to include assessment of UCP gene expression in a variety of other metabolically relevant tissues in relation to winter acclimation, particularly if data could be collected during colder winters.

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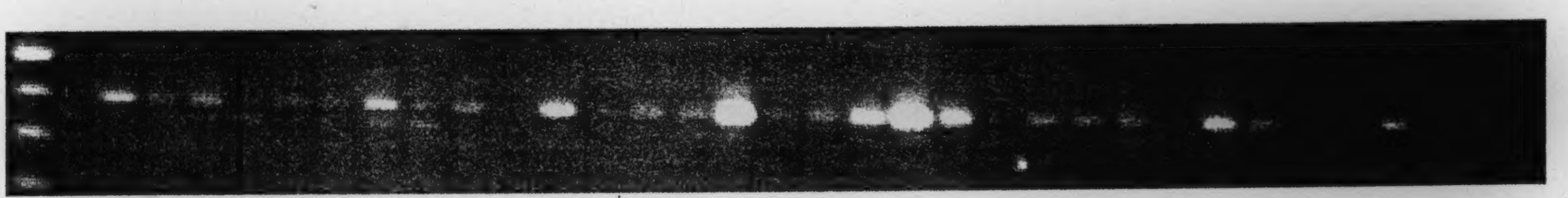

Figure 5-1 UCP3 (top 499b.p) amplicon in *biceps femoris* biopsy with G3PDH (bottom 480 b.p) amplicon as internal control run on a 2% (w/v) Agarose Gel, each lane represents an individual animal. First lane is 100 bp molecular weight marker.

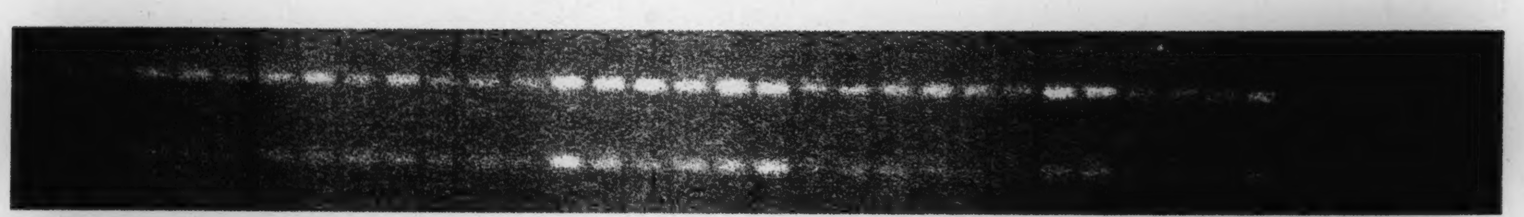

Figure5-2 UCP2 (bottom 263bp) amplicon in *biceps femoris* biopsy with G3PDH (top 480bp) as internal control run on a 2% (w/v) Agarose Gel, each lane represents an individual animal. First lane is 100 bp molecular weight marker.

Figure 5-3 Leptin receptor amplicon (bottom 379 bp) in *biceps femoris* biopsy with G3PDH internal control (top 480 bp) run on a 2% (w/v) Agarose Gel, each lane represents an individual animal. First lane is 100 bp molecular weight marker.

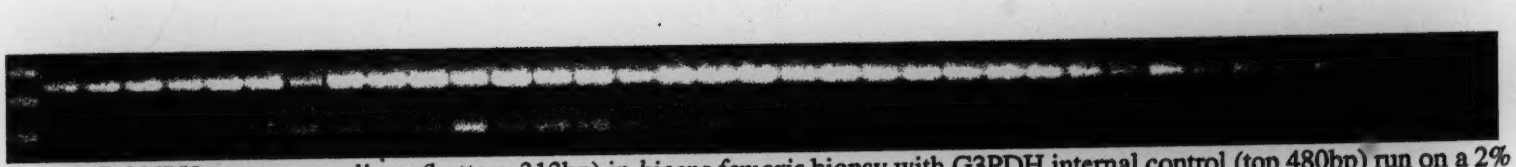

Figure 5-4 NPY receptor amplicon (bottom 313bp) in *biceps femoris* biopsy with G3PDH internal control (top 480bp) run on a 2% (w/v) Agarose Gel, each lane represents an individual animal. First lane is 100 bp molecular weight marker.

Table 5-1 Effect of diet and feeding level on parameters of energy intake, excretion and energy balance in steers.

	Diet and Level of Feeding					Comparisons (p values)			
	Concentrate		Roughage		SE[1]	Diet	Level	D x L[1]	Period
	1.2M[1]	2.2M[1]	1.2M	2.2M					
DEI[1]	0.64	1.06	0.63	1.00	0.02	0.02	<0.0001	0.24	0.43
MEI[1]	0.57	0.99	0.53	0.89	0.02	<0.0001	<0.0001	0.06	0.85
ER[1]	0.05	0.30	0.005	0.22	0.02	0.004	<0.0001	0.38	0.43
HP[1]	0.52	0.69	0.53	0.66	0.01	0.54	<0.0001	0.31	0.14
CH4[1]	2.62	2.02	3.77	4.86	0.19	<0.0001	0.20	<0.0001	0.09
UL[1]	1.94	2.36	1.78	2.25	0.21	0.53	0.04	0.89	0.02
Mem[1]	0.51		0.52		0.016	0.78			
EFME[1]		64.5		54.6	0.06	0.07			

[1] Abbreviations: DEI = Digestible energy intake per kilogram metabolic body weight per day (MJ/kg $^{.75}$/d), MEI = Metabolizable energy intake per kilogram metabolic body weight per day (MJ/kg $^{.75}$/d), ER = Energy retention per kilogram metabolic body weight per day (MJ/kg$^{.75}$/d), HP = Heat production per kilogram metabolic body weight per day (MJ/kg $^{.75}$/d), CH4 = Methane energy loss per day (MJ/d), UL = Urinary energy loss per day (MJ/d), Mem = Maintenance energy requirement per kilogram metabolic body weight per day (MJ/kg $^{.75}$/d), EFME = Efficiency of metabolizable energy use above maintenance (%), SE = Standard error, DxL = Diet x level, 1.2M = 1.2 times the maintenance energy requirement, 2.2M = 2.2 times the maintenance energy requirement.

Table 5-2 Effect of environment on energy intake and balance in steers.

	Environment				Comparisons (p-values)
	Indoor	SE[1]	Outdoor	SE[1]	Environment Difference
DEI[1]	0.82	0.03	0.84	0.03	0.74
MEI[1]	0.74	0.03	0.75	0.03	0.75
ER[1]	0.16	0.02	0.14	0.02	0.63
HP[1]	0.58	0.01	0.61	0.008	0.14
CH4[1]	3.14	0.45	3.31	0.37	0.58
UL[1]	1.71	0.10	2.33	0.08	0.18
MR[1]	0.50	0.02	0.54	0.01	0.12
EFFME[1]	57.7	6.06	57.4	3.60	0.97

[1] Abbreviations: DEI = Digestible energy intake per kilogram metabolic body weight per day (MJ/kg $^{.75}$/d), MEI = Metabolizable energy intake per kilogram metabolic body weight per day (MJ/kg $^{.75}$/d), ER = Energy retention per kilogram metabolic body weight per day (MJ/kg $^{.75}$/d), HP = Heat production per kilogram metabolic body weight per day (MJ/kg $^{.75}$/d), CH4 = Methane energy loss per day (MJ/d), UL = Urinary energy loss per day (MJ/d), MR = Maintenance energy requirement per kilogram metabolic body weight per day (MJ/kg $^{.75}$/d), EFME = Efficiency of metabolizable energy use above maintenance (%), SE = Standard error, DxL = Diet x level

Table 5-3 Effect of environment on heat production in steers during periods one (January) and one (February) in same years, showing progressive changes over time in the different environments.

Experiment and Period	Environment				P values
	Indoor	SE	Outdoor	SE	Environment Difference
1999, period 1, HP[1]	0.58	0.02	0.59	0.01	0.57
1999, period 2, HP[1]	0.58	0.02	0.63	0.01	0.04

[1] Abbreviations: HP = Heat production per kilogram metabolic body weight per day (MJ/kg$^{0.75}$/d), SE = Standard error.

Table 5-4 Composition of diets fed during experiment two.

	Diets[1]			
Composition	**Roughage Based**		**Concentrate Based**	
Ingredients, % of DM				
Alfalfa-grass hay	75		10.0	
Barley grain	23.4		79.4	
Corn gluten meal	-		6.0	
Canola oil	0.6		2.2	
Limestone	-		1.3	
Fortified salt[2]	0.35		0.35	
Coccidiostat[3]	0.23		0.23	
Dynamate[4]	0.1		0.25	
Calcium phosphate	0.25		0.20	
Chemical, % of DM	Period 1	Period 2	Period 1	Period 2
CP	11.6	11.9	13.8	13.4
NDF	54.6	58.9	26.2	28.7
ADF	31.0	31.0	15.5	17.0
Lignin	6.5	5.8	3.0	2.8
DM	91.7	91.7	91.5	92.0
GE, kJ/g	17.5	17.4	17.2	17.4

[1] Rough = Roughage (25 %), Conc = Concentrate (90 %)

[2] Fortified salt = 95% NaCl, 150 ppm I, 50 ppm Co, 3500 ppm Cu, 10000 ppm Mn, 9000 ppm Zn, and 75 ppm Se.

[3] Coccidiostat contained 10,000,000 IU Vitamin A, 1,000,000 IU Vitamin D and 10,000 IU Vitamin E and 330 ppm sodium monensin

[4] Dynamate = 22 % S, 18% K and 11% M

Table 5-5 Correlation's between gene expression analyses in muscle biopsies and indices of efficiency and heat production

	Period 1				Period 2			
	Leptin receptor biceps femoris	**NPY receptor biceps femoris**	**UCP-2 biceps femoris**	**UCP-3 biceps femoris**	**Leptin receptor biceps femoris**	**NPY receptor biceps femoris**	**UCP-2 biceps femoris**	**UCP-3 biceps femori**
Metabolic parameter								
Heat production	-0.262	**-0.347**	0.171	-0.276	0.216	**-0.402**	-0.101	-0.097
MEI	0.108	0.145	0.322	0.078	0.102	0.119	-0.196	0.168
DMER/DMEI	-0.335	-0.266	0.182	0.098	0.040	0.060	0.040	-0.110

N=24, correlation values shown

Table 5-6 Treatment Mean Leptin receptor mRNA abundance[z] in skeletal muscle biopsies; interaction between period 1, 2, 3 & 4 environment and diet.

Environment	Diet type	Mean leptin receptor mRNA	pooled SEM
Inside(Periods1,2,3&4)	concentrate	610.35[1]	25.29
Inside(Periods1,2,3&4)	roughage	630.99[1]	25.29
Outside(Periods1&2)	Concentrate	649.48[1,2]	35.84
Outside(Periods1&2)	roughage	552.97[1,2,3]	35.84

1 Significant difference ($p<0.05$) between environments
2 Significant difference ($p<0.05$) between diet type
3 Significant difference ($p<0.05$) environment*diet interaction
[z] arbitrary densitometric units mean values

Table 5-7 Muscle biopsy gene expression averages^{z} for inside, outside, roughage and concentrate treatments.

	Leptin receptor biceps femoris	**NPY receptor biceps femoris**	**UCP-2 biceps femoris**	**UCP-3 biceps femoris**
Treatment				
Inside average (Period 1&2)	461.28	754.47	586.86	562.63
Outside average (Period 1&2)	443.08	628.75	585.10	579.51
SE	39.50	24.55	7.25	8.24
Probabilities inside vs outside (pvalues)	0.59	**0.06**	0.78	**0.02**
Concentrate average (Period 1, 2, 3&4)	535.33	686.69	590.06	570.63
Roughage average (Period 1, 2, 3&4)	528.40	695.98	581.32	571.52
SE	54.47	32.00	6.72	7.65
Probabilities concentrate vs roughage (pvalues)	0.86	0.68	0.16	0.87

^{z} arbitrary densitometric units

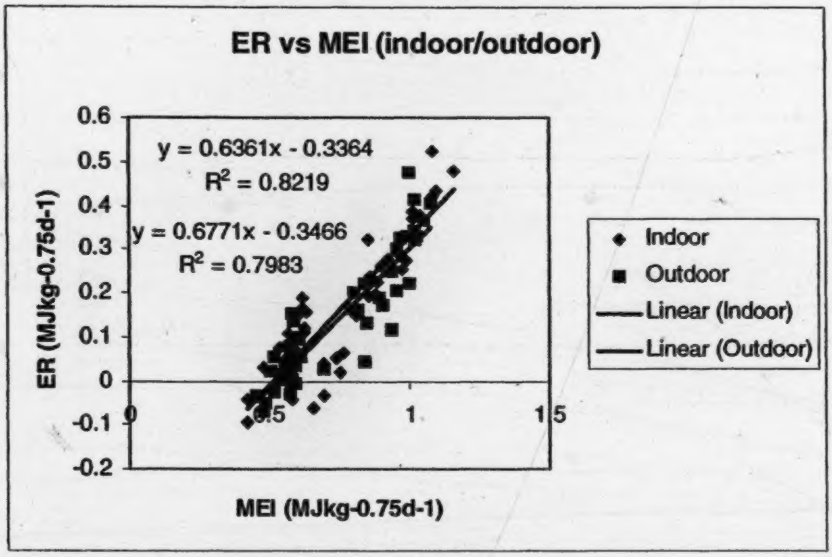

Figure 5-5 Relationship between daily energy retention (ER) and metabolizable intake (MEI) in steers housed in two environments during winter.

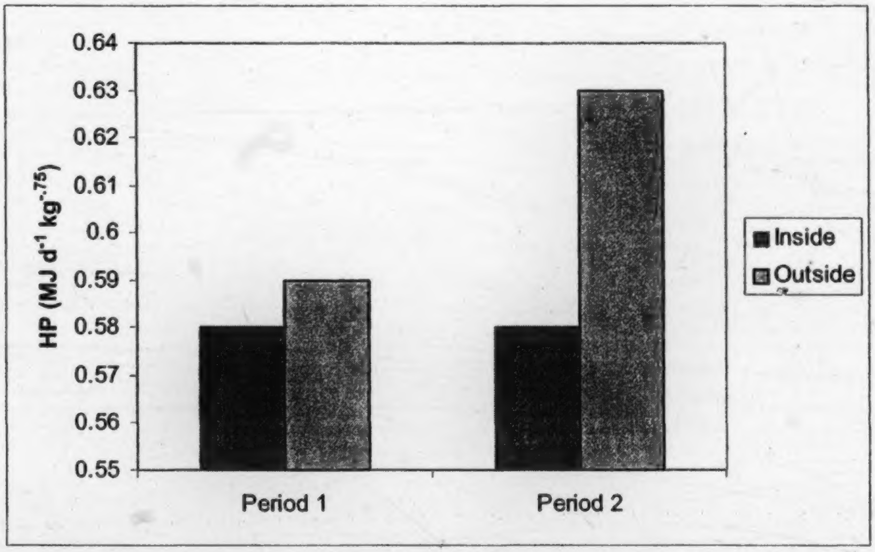

Figure 5-6 Comparison of mean heat production values for inside and outside housed animals over two consecutive periods. Heat production values were significantly different by period 2 following long-term acclimation when inside and outside groups were compared. Period 2 heat production values were significantly different ($p<0.05$) between inside and outside groups.

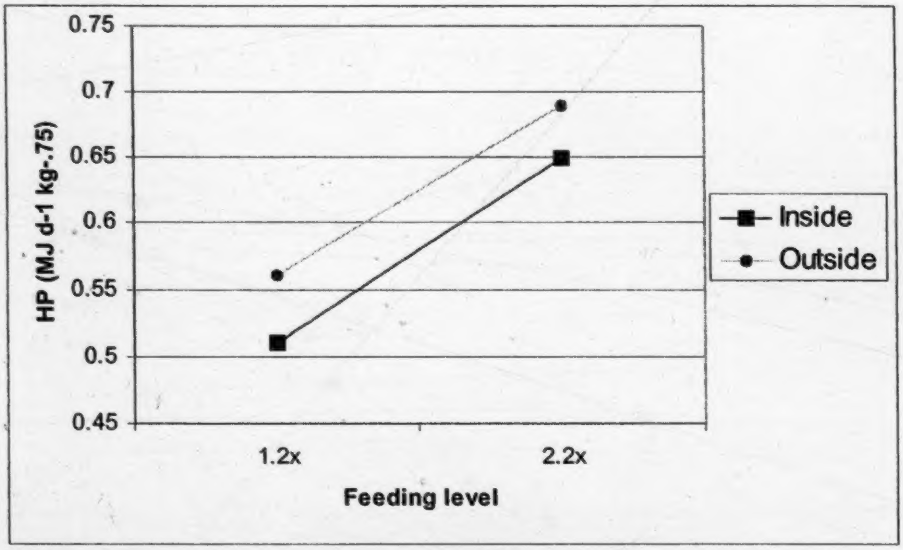

Figure 5-7 Comparison of heat production values over periods 1 &2 for two planes of intake. Increase in heat production in winter acclimated outside steers was higher at both above maintenance planes of intake.

CHAPTER 6

The Effect of Acute Cold Exposure and Acute Feeding Restriction on mRNA Expression in Bovine Tissues

6.1 Introduction

An animal's adaptational strategy in response to an acute stressor is of key importance in determining the effectiveness of that animal's ability to maintain physiological homeostasis. Physiological parameters such as blood pH, blood glucose level and body temperature are not static but rather have a range of acceptable levels, though this range may be narrow, depending on the given physiological state. Therefore, it is perhaps best to consider livestock as homeorrhetic as well as homeostatic. Ideally an animal's response to an acute stressor would be rapid and just sufficient to manage and adapt to maintain a homeorrhetic state. In order to evaluate the response of steers to the acute stress of either moderate cold exposure (-20°C) or partial feed restriction (0.4X maintenance), two scenarios relevant to the Canadian production system, this study examined the animal's response in terms of altered mRNA expression of uncoupling proteins 2 & 3 as well as leptin, leptin receptor and NPY receptor mRNA expression. The protein products of the aforementioned genes are influential in observed cellular and systemic metabolism, in many animal species including humans, though studies in ruminants are limited.

Leptin is often referred to as the systemic lipostat or satiety hormone and as such is an important mediator of nutrient intake and cellular metabolism.

Leptin receptor gene mutations are associated with propensity for obesity such as in db/db mice, fa/fa rats, and it was shown that a leptin receptor gene polymorphism in humans is positively associated with increased adiposity (Yiannakouris et. al., 2001). This is expected since any reduction in sensitivity towards the effects of leptin in terms of its role in satiety signaling (Ahima et. al., 1996), would be expected to result in higher prospective intakes, energy storage and inevitably adiposity. Furthermore, tissue specific leptin receptor gene expression varies between well-fed and feed-restricted ewes (Dyer et. al., 1997) thus it is reasonable to hypothesize a similar scenario in cattle. Neuropeptide Y receptor expression also varies in accordance with fasting in mice and moreover results suggest that general changes in energy balance affect tissue specific NPY receptor expression (Zammaretti et. al., 2001). Given the importance of the physiological pathways that influence feed intake and or nutrient satiety such as that mediated by leptin and neuropeptide Y, variability in tissue specific receptor expression specific for these ligands is likely to be important to an individual steer's regulatory mechanism for nutrient intake and metabolism.

Hyperphagia that is induced by cold exposure has been linked to variation in the plasma leptin concentration in rats (Bing et. al., 1998), which further emphasizes the importance of the leptin/NPY nutrient sensing pathway in adaptive strategies related to maintaining energy balance. Since the effects of leptin and neuropeptide Y not only include energy balance but also influence cellular metabolism, we hypothesized that the tissue-specific expression of mRNA for the receptors of these ligands may vary between individual animals and tissues. It was further hypothesized that the tissue-specific expression of these genes would be indices of the status of this nutrient sensing pathway as well as an indicator of its typical adaptation upon exposure to the stress of acute feed-restriction and acute cold exposure in steers.

Some studies in rats have shown that increases in skeletal muscle UCP-2 (and UCP-3) are proportional to increased measures of hyperthermia and mitochondrial proton leak kinetics (Yu et. al., 2000). Therefore in scenarios where increased thermogenic capacity is advantageous, such as during cold exposure, it would be a useful strategy to increase uncoupling protein gene expression so as to invoke elevated cellular heat production. Support for the hypothesized role of UCP-2 in adaptive thermogenesis is conveyed by its consistent upregulation in response to cold that is observed in a broad range of studies, including those in chickens (Raimbault et. al., 2001), hummingbirds (Vianna et. al., 2001), and even a UCP homologue in plants (Pecquer et. al., 2001), but not yet in cattle other than UCP-1 in brown fat (Carstens et. al., 1996).

There also appears to be nutrient mediated regulation of UCP-2 expression, for instance lipids can activate the UCP-2 gene transcription. It has been reported that isoenergetic substitution of carbohydrate with fat resulted in increased expression in rat muscle, though thermogenesis was lower in the high fat than the low fat diet animals (Samec et. al., 1998). UCP-2 is positively correlated with plasma insulin levels, except in individuals with non-insulin dependent diabetes mellitus (NIDDM) (Vidal et. al., 1999). Uncoupling protein-2 is consistently elevated in skeletal and white adipose of acute fasted rats (Pinkney et. al., 2000) and humans (Millet et. al., 1997) and also when hypophagia is induced as in MAC-16 induced cancer cachexia (Bing et. al., 2000). However this relationship is again abnormal in skeletal muscle of type-2 diabetics, where UCP-2 mRNA and protein are unchanged by acute fasting. The story becomes more complex when one considers that UCP-2 expression in white adipose is increased by chronic leptin treatment, but down-regulated during acute leptin treatment (Combatsiaris and Charron, 1999). Though this still supports the idea that reduced UCP-2 expression may be a maladaptive response to sustained energy surplus and that this may be relevant to the onset and maintenance of obesity and obesity-related disease such as diabetes.

As mentioned, under conditions of fasting UCP-2 is up-regulated, as would be the rates of lipolysis, this increase in UCP is in contrast to what would be expected if UCP's sole function were as a positive regulator of thermogenesis. A more recent study involving T3 treatment of mice found a good correlation between both UCP-2 and UCP-3 up-regulation in skeletal muscle and increased resting metabolic rate (Jekabsons et. al., 1999). Boss et. al. (1998) re-iterated that 2 day cold exposure failed to elevate UCP-3 expression in rat skeletal muscle but that 1 week feed restriction (50%) decreased UCP-3 by 81% while acute 24 hour fasting elevated UCP-3 transcripts 6 fold (Boss et. al., 1998). In transfected myoblasts, Boss et. al. also validated the capacity for UCP-3 to uncouple myoblasts (1998). These results suggest that UCP-3 expression may be more important in the regulation of energy expenditure, body weight, metabolic rate and nutrient sensitive metabolic adaptation rather than cold-induced thermogenesis.

The study described in this chapter was therefore designed to test the hypotheses that the expression of uncoupling proteins 2 and 3, leptin, leptin receptor and neuropeptide Y receptor in various bovine tissues may be influenced by acute cold and acute feed restriction.

6.2 Materials and methods

6.2.1 Animals and Management

Thirty eight steers (36 experimental plus two spares) of mixed breeding (BW ± SD, 204 kg ± 20 kg) were brought from the University of Alberta Ranch, Kinsella, Alberta to the Laird McElroy Environment and Metabolism Center located at the Edmonton Research Station, Edmonton, Alberta. Upon arrival the steers were fed in groups and had constant access to water throughout the following adaptation periods. The steers were housed outside in pens and were initially fed a diet consisting of long stem hay (alfalfa/brome grass mix) and rolled barley (50:50 ratio) for a period of 2months. Steers were then switched to a backgrounding diet and utilized as experimental subjects of an *ad libitum* roughage and *ad libitum* concentrate trail (results to be submitted). When the steers reached 480kg± 41kg they were placed on a finishing diet comprised of 90 % (w/w) concentrate 10% (w/w) roughage, for a further 6 weeks prior to commencement of the acute study. Animals were weighed before they were placed on the feeding regimen and at two weeks intervals.

6.2.1.1 *Effects of Acute Cold Exposure or Feed Restriction on Gene Expression in Steers.*

Comparisons were made of 2.2X maintenance feeding in a thermoneutral environment with either a 2.2X maintenance feeding during acute cold (-20°C) exposure or feed restriction down to 0.4X maintenance in a thermoneutral environment prior to slaughter, on relative rates of gene expression, as measured by gene-specific mRNA detection. Treatments were imposed for four days just prior to slaughter. *Post-mortem* tissue samples were taken and snap-frozen for broad-spectrum analyses of treatment effect on gene expression profiles in internal and external tissues.

Carcass grades were also obtained for additional comparison with the treatment and tissue-specific gene expression values.

6.2.2 Gene Expression Studies

6.2.2.1 RNA Extraction

Total RNA was extracted from measured quantities of frozen powdered tissue samples for each individual animal using a modified Chomczynski protocol (1993) and Gibco Trizol™ phenol-chloroform phase extraction procedure. The integrity of each Total RNA sample was examined using electrophoresis on agarose gels and examination of the abundance and integrity of the 18S and 28 S ribosomal RNA bands. The total RNA purity and quantity were determined for each sample using absorbance measurements at wavelengths of 260nm and 280nm, and the respective ratio of these measures. Working stocks (50μl) of 1μg total RNA/μl for each sample were prepared using DEPC-treated water. These working stocks were utilized for the reverse transcription and subsequent PCR analyses.

6.2.2.2 *Reverse Transcription and Polymerase Chain Reaction*

Reverse transcription was performed for each sample using 1ug total RNA, oligo-dT^{15} primer and Gibco MMLV-RT enzyme, in the presence of RNase inhibitor (Gibco RNAase OUT) at 37 ° C for 60 minutes in a thermocycler (Perkin Elmer 2400 or Biorad icycler). Additional details regarding the reverse transcription protocol are included in the Appendix. Each reverse transcription reaction supplied template for a minimum of three specific multiplex PCR reactions using specific primer sets and Taq polymerase enzyme (Sigma). Sense and antisense primer sets were designed for each of the specific target gene using Genbank as a resource for specific bovine gene and mRNA sequences. The optimized primer sets are able to specifically amplify and thus semi-quantify bovine mRNA representing the following genes; leptin, leptin receptor, neuropeptide Y receptor, uncoupling protein 2 (UCP2), uncoupling protein 3 (UCP3). Further, we have utilized a primer set for a constitutively expressed gene; glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as the internal control in each of the specific relative reverse transcription polymerase chain reactions (relative RT-PCR). This internal control normalizes for slight variations in reaction efficiency, RNA loading and pipetting. The forward and reverse primer sequences are included in the appendix as are the amplicon sizes for each specific gene. All PCR reactions were performed on either a Perkin Elmer 2400 thermocycler or BioRad Icycler. The PCR amplicons were electrophoresed on a

2% (w/w) agarose /TBE gel along side a molecular weight marker (Gibco 100bp DNA ladder). The gels were stained using ethidium bromide (0.1% w/v) so as to observe the PCR products under UV fluorescence. The specific amplicon bands could thus be relatively quantified by generating optical density scores for the amplicon bands using Biorads Gel Doc and Molecular Analyst software.

The RT-PCR reactions were validated for linearity with regards to total RNA quantity and cycle number and the amplicons were sequenced to validate the specificity and selectivity for the genes.

6.2.3 Statistical Analyses

All data were analyzed using SAS, PROC GLM, least square means analyses with Standard error of the mean.

6.3 Results

6.3.1 Gene Expression Profiles

6.3.1.1 Leptin, Leptin Receptor and NPY Receptor Message

Figures 6-1, 6-2, and 6-3, show typical images of specific gene PCR amplicons separated on 2% (w/v) agarose gels, allowing for relative abundance determination by optical density measurement and relative comparison to the internal control amplicon. Table 6-1 summarizes mRNA data measured in two internal tissues and biceps femoris muscle collected at slaughter from steers in control (thermoneutral, 2.2M feeding), acute restriction (thermoneutral, 0.4M feeding) and acute cold (-20°C, 2.2M feeding) exposure. There were no effects of either acute cold exposure or acute feed restriction on leptin, leptin receptor, and

NPY receptor mRNA values in *biceps femoris* muscle. The leptin receptor expression in the duodenum tended to increase (p=0.06) in the feed restricted animals (553.5 ± 12.35) as compared to the 2.2X maintenance controls (519.2± 12.35)

The leptin receptor mRNA expression in the liver was significantly increased (p=0.02) by feed restriction, whereas the NPY receptor mRNA expression in the liver was significantly decreased (p=0.01) by the feed restriction. Although the cold stress did not significantly alter the leptin receptor mRNA in the liver, the NPY receptor mRNA in the liver was significantly decreased (p=0.03) in the cold treated steers versus the thermoneutral controls.

The NPY receptor mRNA expression in the mesenteric adipose tissue was not significantly altered by feed restriction but was significantly increased (p=0.03) by acute cold treatment. Moreover, there was an apparent trend for the leptin receptor mRNA in mesenteric adipose tissue to be decreased by acute cold stress that failed to reach significance (p=0.18).

It was further observed that there was a disparity in the leptin mRNA when comparisons were made between cold exposed subcutaneous adipose tissue and peri-renal adipose tissue (Table 6-2). The effect of cold exposure lowered the observed leptin mRNA present in sub-cutaneous adipose tissue (p=0.04), while the leptin mRNA present in peri-renal adipose tissue of cold exposed steers tended to be higher than that of the thermoneutral controls (p=0.09).

This study further examined the mRNA expression of UCP-2 and UCP-3 in the selected tissues in the context of our acute treatments (Table 6-3). It is important to note that the expression of UCP-3 mRNA was not significantly altered by the acute cold and feed restriction treatments. This may reflect the duration and timeline of treatment and/or the timeline for tissue sampling, but regardless, the UCP-3 mRNA was not altered in treatment steers versus controls.

In stark contrast to the UCP-3 mRNA, the measured UCP-2 mRNA levels in both subcutaneous adipose tissue and *biceps femoris* skeletal muscle were significantly changed by both acute cold and acute feed restriction. The average UCP-2 mRNA in the subcutaneous adipose tissue of cold treated steers was significantly greater (p=0.0004) than thermoneutral control steers (table 6-2). Additionally, the feed restriction also resulted in significant increase in measured UCP-2 mRNA in the subcutaneous adipose tissue (p=0.03). A similar increase in UCP-2 mRNA was observed in *biceps femoris* tissue during acute cold (p= 0.003) and feed restricted (p=0.002) in comparison to the expression in *biceps femoris* tissue of control steers.

The current study further revealed some interesting results upon comparison of the carcass grades obtained for the 36 cattle, assessed by a trained and experienced abattoir grader, with the tissue-specific mRNA data. In particular, there was a significant association (p=0.006) between the carcass grading score of A, AA, or AAA and the mean leptin mRNA in the subcutaneous adipose tissue samples for carcasses belonging to each group (Figure 6-4). The respective means for A, AA and AAA animals subcutaneous adipose tissue mRNA were 653.40 ± 61.02, 535.49± 13.72, and 501.56± 29.87. This may suggest that a decrease in leptin mRNA expression in subcutaneous adipose tissue is associated with a higher carcass grade in beef cattle that grade either A, AA, or AAA.

6.4 Discussion

The association of neuropeptide Y with appetite has been established for much longer than it has been known regarding leptin. In 1984, research showed that central administration of NPY in rats could induce excessive eating (Stanley et. al., 1985). Furthermore, in line with the up-regulation of NPY during acute fasting it has also been established that NPY mediates the initiation of reduced energy expenditure in an attempt to conserve energy. This was shown by Billington et. al. (1991) who observed a decrease in brown fat thermogenesis and UCP-1 expression in centrally-infused NPY mice. NPY appears to be the flip-side of the coin, as compared to leptin, in terms of their individual effect on both nutrient intake and energy expenditure. Prolonged central administration of exogenous NPY has been shown to promote adipose accumulation and ultimately result in obesity (Stanley et. al., 1986; Zarjevski et. al., 1993).

Regulation of NPY is also affected by ambient temperature as acute cold exposure (2.5-18 hours) reduced NPY, allowing the sympathetic activation of brown fat, increased UCP and thus elevated thermogenesis (Bing et. al. 1996). In contrast, longer term cold exposure was found to elevate NPY and is hypothesized to support the increase in dietary intake associated with cold (Kotz et. al., 1998).

In a recent experiment, starvation decreased the Y1 receptor for NPY in the PVN but not in the arcuate nucleus, but treatment of drinking water with 10% (w/v) glucose resulted in elevated Y1 receptor expression in both the PVN and the arcuate nucleus (Zammaretti et. al., 2001). Additionally, the Y1 receptor subtype mRNA was up-regulated in association with transient hyperphagia and body weight gain which was suggested to represent evidence for the hypothalamic mediated development of leptin resistance (Kalra, et. al., 1998).

The statistical analyses of the gene expression profiles revealed several effects of our acute treatments related to ambient temperature and feed restriction. The significant reductions in NPY receptor gene expression in liver, due to both acute cold exposure (p=0.025) and acute feed restriction (p=0.01) (Table 6-1) may be an indication of stressed animals preparing for reduced nutrient availability for storage if for instance more of the metabolizable energy is diverted towards heat production in the cold or maintenance in the feed-restricted steers. It may also represent a relationship between liver NPY receptor expression and stress-related events such as elevated ACTH or cortisol, but more studies would be required to discern the observed receptor modulation. An additional observation regarding NPY receptor mRNA, was that in mesenteric adipose tissue there appears to be an increase in association with acute cold exposure (p=0.03). This emphasizes the importance of examining tissue-specific gene expression as we observe an increase in the mesenteric adipose NPY receptor mRNA while there is in contrast a significant decrease in the NPY receptor mRNA in the liver upon acute cold treatment. This apparent disparity may in fact emphasize the dual role of NPY, involved in both nutrient intake and partitioning as well as cellular metabolism. As mentioned earlier, Bing et al. (1996), noticed a drop in NPY after acute cold exposure, which they hypothesized would facilitate thermogenesis, followed by an increase in NPY with chronic cold to facilitate hyperphagia. One may extend this hypothesis to suggest that the decrease in NPY may result in the tissue-specific increase in mesenteric adipose NPY receptor specific mRNA. This would facilitate the transition towards more chronic adaptation of cold-induced hyperphagia, in response to subsequent NPY increases and specifically promote nutrient uptake and storage in the mesenteric adipose tissue. The exact timeline in terms of acute or chronic adaptational strategies remain to be determined in cattle as do the extent of adaptation and the range of ambient temperatures for which the adaptation is observed. This study provides support for more research regarding tissue-specific NPY receptor expression, which would include protein quantification in addition to further mRNA measurement.

Many publications related to research performed on animal species other than cattle, led to the hypotheses that tissue-specific leptin receptor mRNA expression would also be important in the bovine. It was expected that the leptin receptor mRNA would be present in many tissues collected from steers just as was shown with mass screening of mouse tissue using RT-PCR and RNase protection assay. The leptin receptor (OB-Rb), was found to be present in the mouse lung, heart, liver, spleen, various WAT depots, skin, bone, brain cortex, pituitary, adrenals, stomach, small intestine, colon, kidneys, bladder, testis and hypothalamus (Lollman et. al., 1997). Therefore, it is probable that leptin exerts some influence on peripheral tissues outside the central mediation of satiety and that this is likely to involve its specific receptor and its relative expression on the cell surface.

The significance of the leptin receptor to the leptin-mediated regulation of nutrient intake is emphasized by the db/db obese mice and the fa/fa obese rats but also by other studies. For instance, a spontaneous mutation resulted in an obese and hypertensive rat that was determined to have a null leptin receptor mutation (Wildman et. al., 2000). Furthermore, a single nucleotide polymorphism in the leptin receptor of postmenopausal Caucasian women has been well correlated with BMI, fat mass and leptin levels indicating that it may represent a scenario of reduced insulin sensitivity (Quinton et. al., 2001). Very limited investigation of the leptin receptor in cattle had been reported prior to our study. This study's results in the context of acute nutrient restriction and its effect on tissue-specific leptin receptor mRNA abundance are the first of their kind in cattle, and point out some interesting complexities in the role of leptin in metabolic regulation in cattle

The eventual impact on energy metabolism of a reduction in NPY receptor gene expression, and increases in leptin receptor gene expression in both the liver (p=0.02) and the duodenum (p=0.06) in response to acute feed restriction (Table 6-1) are uncertain. However, if one considers how important changes in metabolic parameters in the digestive tract and organs such as the liver may be in terms of whole animal efficiency and metabolic adaptation in response to potential stressors such as cold exposure, then the current data suggests involvement of these genes in gut activity. This opens some potentially important avenues for further research quite apart from central nervous signaling of hunger and satiety.

The regulation of leptin receptor gene expression will likely prove to be an important factor in the orchestration of the normal balance between orectic and anorectic signals. It is postulated that NPY itself suppresses leptin receptor expression and this is supported in principal by the elevated OB-Rb expression in NPY knockout mice (Baskin et. al., 1998). It is also suggested that the activation of the leptin receptor itself act as an autoregulator of its gene expression as induction of the "Suppressor of Cytokine Signaling (SOCS)" may suppress leptin receptor gene transcription (Bjorbaek et. al., 1998). It will become increasingly important to elucidate these physiological processes in ruminants as we discover just how important and complex the orectic and satiety signals are in terms of their modulation of not simply nutrient intake but systemic and peripheral tissue cellular metabolism, as well.

When similar analyses were performed on the gene expression profiles in adipose depots of the acutely feed-restricted steers, surprisingly no significant adaptation of leptin gene expression was observed (Table 6-2). There was however a significant effect of acute cold stress on the leptin mRNA abundance in both subcutaneous (p=0.04) and perirenal (p=0.09) adipose tissues. Whereas the effect of cold stress decreased the leptin mRNA abundance in the subcutaneous adipose tissue, the opposite was true in peri-renal adipose tissue as the leptin mRNA abundance increased in the acute cold exposed steers.

The lack of a significant adaptation of leptin gene expression in adipose tissue in response to feed restriction is perhaps surprising given the results of studies on other animal species. On the otherhand, the significant increase in leptin mRNA in subcutaneous adipose tissue, and the significant decrease in the expression of this gene in perirenal adipose tissue, suggests that pelvic depots respond differently to cold exposure.

The UCP-2 and UCP-3 transcripts detected in the *post-mortem* tissues (Table 6-3) were similar to other screened values for candidate genes in that the key consistency appears to be the individual animal variation. Without further investigation it is difficult to hypothesize why these variations are present. It may indicate inherent differences in the animals, or acute or fluctuating regulation of the expression of these genes. It is also possible that the intrinsic turnover rates for the products of these genes may necessitate such animal variation in gene transcription. Regardless of the underlying cause of the observed variability it emphasizes the physiological differences between these growing steers.

Although the results of this current study did not indicate any significant changes in UCP-3 mRNA abundance that were attributable to acute treatments, the data shows that there are highly significant ($P< 0.003$) changes in UCP-2 expression in *post-mortem* samples from acutely cold-exposed and feed-restricted steers. Changes suggest that mean UCP-2 mRNA levels increase in the most peripheral tissues such as subcutaneous adipose and *biceps femoris* skeletal muscle, in response to acute cold-exposure. These findings indicate that in peripheral adipose tissue and its underlying skeletal muscle, those tissues potentially most affected by cold external ambient temperatures, adaptation of UCP-2 mRNA expression occurs in accordance with its potential for increasing thermogenic capacity. Linkage between normal thermoregulatory events such as increased peripheral vasomotor tone (PVMT) and thermogenesis and increased UCP-2 expression may all be part of the compensatory adaptation. One might expect that this up-regulation of UCP-2 may assist in non-shivering thermogenic capacity in these steers, as a result of oxidative phosphorylation becoming more loosely coupled to ATP synthesis. These findings are supported by other research showing cold-induced increases in UCP expression (Rehnmark et. al., 1992, Milner et. al., 1989). Although an increase in muscle and subcutaneous UCP-2 expression may represent a decrease in energetic efficiency in these animals, the ability to adapt to increase thermogenic capacity in response to cold is essential in the maintenance of healthy cattle in Canadian livestock production.

Additionally, UCP-2 message was also increased in the skeletal muscle and subcutaneous adipose tissue of acute feed-restricted animals which agrees with findings by Thomas and Palmiter, (1997) and Millet et. al. (1997). This may seem counterproductive in terms of energy conservation, but it may be related to the role of UCP-2 as a protection against accumulation of reactive oxygen species generated in mitochondria of tissues that are increasing their catabolic activity especially lipolysis and fatty acid oxidation. Furthermore, it is well established that free fatty acids can themselves induce UCP-2 gene expression and one would expect that serum free fatty acid concentration would rise in response to feed restriction-induced lipolysis.

One final interesting result of this study emerged when it was ascertained that there appears to be an association between the carcass grades and the abundance of leptin mRNA, specifically in an animal's subcutaneous adipose. This study elucidated a simple correlation where increased leptin gene expression is present in tissue samples of steers that graded lower on the scale of single A, double A, or triple A, carcasses. This may reflect different individual animal maturity, body composition and or metabolic status at the time of slaughter, but additional studies are required to fully discern the significance of this association. Since there have been several reports of associations between leptin gene polymorphisms related to carcass adiposity and parameters like backfat thickness in pigs, perhaps our study serves as supportive evidence that leptin itself may be important in contributing to what one generally considers a better grade of carcass.

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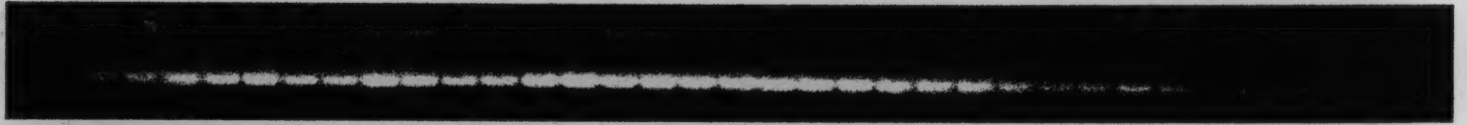

Figure 6-1 NPY receptor amplicon bottom in subcutaneous adipose tissue with G3PDH top, run on a 2% (w/v) Agarose Gel, each lane represents an individual animal. First lane is 100 bp molecular weight marker.

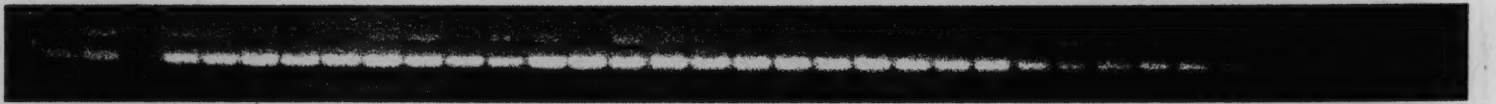

Figure 6-2 Leptin receptor amplicon bottom in subcutaneous adipose tissue with G3PDH top, run on a 2% (w/v) Agarose Gel, each lane represents an individual animal. First lane is 100 bp molecular weight marker.

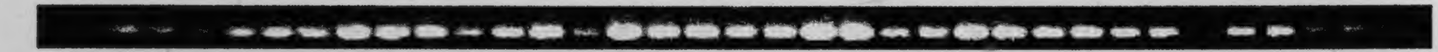

Figure 6-3 Leptin amplicon (460bp) in subcutaneous adipose tissue, run on a 2% (w/v) Agarose Gel, each lane represents an individual animal. First lane is 100 bp molecular weight marker.

Table 6-1 Average and standard deviations of gene mRNA expression^z in acutely treated steers

	Leptin receptor duodenum	NPY receptor duodenum	Leptin receptor liver	NPY receptor liver	Leptin biceps femoris	Leptin receptor biceps femoris	NPY receptor biceps femoris
Treatment							
2.2 M average	519.20	85.11	587.07	365.28	536.12	533.76	572.12
0.4 M average	553.52	80.73	639.37	325.98	542.29	529.69	577.29
Cold average	533.35	85.78	601.99	327.88	567.54	546.72	589.24
SE	12.15	11.07	12.39	11.33	17.09	17.49	34.41
Probability cold (pvalues)	0.39	0.99	0.37	**0.02**	0.24	0.61	0.75
Probability (0.4M) (pvalues)	0.06	0.80	**0.02**	**0.01**	0.78	0.87	0.91

^{z} arbitrary densitometric units, mean values reported

Table 6-2 Average and standard error of gene mRNA expression^z in acutely treated steers

	Leptin mesenteric adipose	**Leptin receptor mesenteric adipose**	**NPY receptor mesenteric adipose**	**Leptin subcutaneous adipose**	**Leptin receptor subcutaneous adipose**	**NPY receptor subcutaneous adipose**	**Leptin perirenal adipose**	**Leptin receptor perirenal adipose**	**NPY receptor perirenal adipose**
Treatment									
2.2 M average	102.42	900.63	477.21	566.28	784.26	827.16	620.94	317.57	110.73
0.4 M average	99.93	882.74	486.77	554.06	796.75	835.80	641.65	310.26	111.46
Cold average	111.20	864.88	500.01	534.78	788.95	797.32	647.76	318.88	111.70
SE	4.48	16.65	8.25	18.16	15.23	20.63	9.18	5.98	6.78
Probabilities cold (pvalues)	0.51	0.18	**0.03**	**0.04**	0.82	0.38	0.09	0.89	0.94
Probabilities restricted (pvalues)	0.87	0.55	0.39	0.62	0.53	0.80	0.39	0.44	0.95

^{z} arbitrary densitometric units, mean values reported

Table 6-3 Averages and standard error of gene mRNA expression^z values in *post mortem* tissues from acute *antemortem* treated animals.

	UCP-2 mesenteric adipose	**UCP-2 subcutaneous adipose**	**UCP-2 perirenal adipose**	**UCP-2 liver**	**UCP-2 biceps femoris**	**UCP-3 biceps femoris**
Treatment						
2.2 X average	172.13	464.11	754.65	79.14	790.57	551.29
0.4 X average	176.05	526.27	781.84	90.09	840.32**	545.13
Cold average	165.05	544.89**	765.89	92.30	847.78**	561.09
SE	14.93	12.36	21.09	9.18	11.35	8.49
Probabilities cold (pvalues)	0.74	**0.0004**	0.73	0.24	**0.003**	0.49
Probabilities restricted (pvalues)	0.85	**0.03**	0.29	0.44	**0.002**	0.71

^{z} arbitrary densitometric units, mean values reported

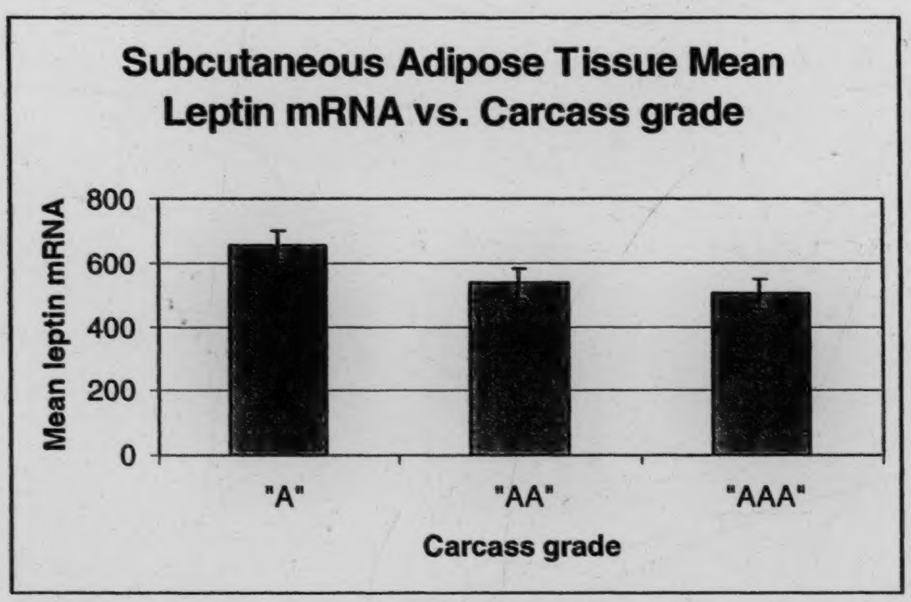

Figure 6-4 Comparison of mean leptin mRNA expression (arbitrary densitometric units) in subcutaneous adipose tissue between carcass grades.

Chapter 7

General Discussion and Conclusion

7.1 General Discussion

With current improvements in molecular biology techniques, and the ability to measure and investigate changes in gene expression, inferred from evaluation of relative mRNA abundance, we have available an important tool for investigation of ruminant physiological processes. More specifically we are now able to ascertain bovine tissue-specific expression of known genes and as well characterize physiological adaptation strategies to production-relevant stressors in the context of changes in these mRNA abundance profiles.

This study represents the most thorough investigation to date, of tissue-specific mRNA abundance of these candidate genes in the bovine species. Moreover, this research is the first broad-spectrum analyses of mRNA abundance in bovine that attempted to characterize potential variability in these genes in the context of breed, plane of nutrition, diet type, and adaptation to industry relevant stressors. The subsequent sections will review some of the general observations that were made throughout this research project in the context of each of our candidate genes.

7.1.1 Uncoupling protein-1 (UCP-1)

The initial screening of our bovine tissues found only trace presence of UCP-1 specific mRNA in a few of our adipose samples. Since, these samples were taken from reasonably young, backgrounder cattle it is possible that these adipose samples may have been contaminated with trace amounts of brown fat in which UCP-1 appears to be most commonly identified. Since the UCP-1 mRNA that we detected was limited, and did not appear to relate to any of our treatments or groups we limited our focus so that this initial screening was the extent of our inclusion of UCP-1 as a candidate gene.

7.1.2 Uncoupling protein-2 (UCP-2)

In stark contrast to UCP-1, our initial screening of UCP-2 isoform-specific mRNA revealed a rather ubiquitous expression profile across different tissue types, although it was not detectable in our total RNA extracted from either duodenal scrapings or the abomasal scrapings. Since UCP-2 has been demonstrated in the small intestine of rats (Murase et. al., 2001), the fact that we did not identify its mRNA message in the duodenal scrapings from our steers may indicate another significant difference between the gastrointestinal tract of ruminants versus monogastric species. On the flip-side of comparison the observation that UCP-2 mRNA was detectable in most of our screened tissues agrees with literature regarding UCP-2's ubiquitous expression in other animal species (Fleury et. al., 1997, Gimeno et. al., 1997, Surwit et. al., 1998, Gong et. al., 1999, van der Lee et. al., 2000).

In general we found that the individual animal variability in the expression of UCP-2 mRNA abundance was such that it masked our identification of between breed differences. Of course, the presence of substantial individual animal differences in UCP-2 mRNA abundance begs the question as to the physiological significance of such variability. When we compared breed-dependent mean UCP-2 mRNA abundance measures from screened rumen papillae we noted that Brangus rumen papillae expressed significantly lower (p=0.006) UCP-2 mRNA than Charolais and Angus steers. The significance and effect of this breed difference remains to be addressed and ascertained. We further observed that the mRNA abundance of UCP-2 in mesenteric adipose tissue also suggested a breed effect difference related to feed intake. Whereas the Brangus and Angus steers showed significant increases (p<0.001) in their relative UCP-2 mRNA abundance when on the 2.2X maintenance plane of nutrition as compared to the lower 1.2X maintenance feeding level, the Charolais steers showed no comparable differences in UCP-2 mRNA attributable to feeding level. It was suggested that diet-induced increases in white adipose UCP-2 mRNA abundance may serve as a component of a compensatory mechanism to prevent the onset of obesity (Surwit et. al., 1998). Although all of our steers were young, growing and of reasonably lean body composition, it was our subjective interpretation that the Charolais steers were perhaps the most lean of our breeds. We may speculate that the Charolais cattle may therefore have had the least to benefit from the initiation of such a compensatory mechanism as suggested by Surwit.

The positive correlation between UCP-2 abundance and metabolic rate has been shown in other species (Bouchard et. al., 1997, Langhans, 2002), and has been attributed to retention of its uncoupling capacity (Fleury et. al., 1997, Ricquier and Bouillard, 2000). Our results indicated a positive correlation (r=0.33) between UCP-2 mRNA abundance in mesenteric adipose tissue and our measures of heat production. We also report that the UCP-2 mRNA is positively correlated with MEI in both *biceps femoris* skeletal muscle (r=0.45) and mesenteric adipose tissue (r=0.33).

Of course, cause and effect are not established by such correlations however they do support the hypotheses that the UCP-2 expression in some metabolically active and abundant tissue may influence the systemic heat production of individual cattle, and in turn may reflect heat production differences that occur in association with increased metabolizable energy intake.

A trend (p=0.15) that indicated a modest increase in skeletal muscle UCP-2 abundance in association with concentrate versus roughage diet type may also be important towards our improved understanding of the complexities of adaptive energy metabolism in livestock. So too the association between UCP-2 mRNA abundance in skeletal muscle and Metabolic body weight (p=0.03), needs to be investigated if we are to discern the context of its physiological significance. This is especially true given that there was an apparent trend for reduced UCP-2 mRNA abundance in skeletal muscle with increased period (perhaps age), which may suggest that a reduced UCP-2 expression with maturity may facilitate the deposition of adipose in this specific tissue which may be quite pertinent to aspects of finishing cattle and marbling.

7.1.3 Uncoupling protein-3 (UCP-3)

The UCP-3 mRNA was far less ubiquitously expressed across our sampled tissues than observed for UCP-2. We found UCP-3 mRNA in our *biceps femoris*, which agrees with studies involving other species that have determined that this isoform is predominantly observed in skeletal muscle (Boss et. al., 1997, Vidal-

Puig et. al, 1997). We also identified varied expression of UCP-3 mRNA in cardiac muscle samples which represents the first such report of its presence in this bovine tissue, though previously reported in human cardiac muscle (Boss et.al., 1997).

Several separate studies have provided evidence that UCP-3 expression is an important component of systemic energy metabolism. For instance, there is a significant linkage between UCP-3 gene location (in humans 11q13), with resting metabolic rate, body mass index, percentage body fat, and overall fat mass (Bouchard et. al., 1997). This finding was corroborated in a study of Pima Indians, also predisposed to obesity and type II diabetes, where a negative correlation was observed between Skeletal muscle UCP-3 and both RMR and BMI (Schrauwen et. al., 1999). We determined that the relative UCP-3 mRNA abundance measured in cardiac muscle was negatively correlated (r = -0.34) with energy retention. Perhaps an increase in cardiac muscle UCP-3 inherently renders the heart less efficient due to its ability to uncouple the proton gradient while at the same time conferring some protection from damage associated with accumulation of reactive oxygen species in this hard working organ. The decrease in energetic efficiency in the cardiac muscle may also translate into a reduction in systemic efficiency and as such negatively impact an animal's retention of energy.

We also noted sporadic UCP-3 mRNA present in some of our peri-renal adipose tissue samples, and this agrees with reports of its presence in adipose of other animal species (Larkin et. al. 1997).

We did not observe any variation in UCP-3 abundance that was attributable to breed, nor were there associations related to feeding level identified in our studies. This latter observation may be related to the modest degree of difference between our feeding levels, or the duration of lower nutritional feeding but may also be a result of our limited sampling times. Previous studies have indicated that there may be dynamic regulation of UCP-3 mRNA abundance that is dependent upon the extent and duration of feed restriction such that 1 week feed restriction (50%) decreased UCP-3 by 81% while acute 24 hour fasting elevated UCP-3 transcripts 6 fold (Boss et. al., 1998).

Additionally, there was a slight trend (p=0.10) towards increased UCP-3 mRNA abundance in skeletal muscle biopsies from our long-term cold acclimated (outside) steers versus our inside housed steers. Given that we were afforded only modestly cold ambient temperatures (~-10°C) during this phase of our trial we might speculate that this observed UCP-3 adaptation may have been more pronounced and in turn more significant under the influence of lower ambient temperatures. The fact that our acute cold treatment (4 days @-20°C) did not result in significant differences in the measured UCP-3 mRNA may allude to a more chronic regulation of this gene's expression that was missed by our sampling timeline. However, the fact that Boss et. al. (1998) also reported an absence of up-regulation of UCP-3 upon a two day cold exposure in rat skeletal muscle might also suggest that UCP-3 is more involved in regulation of longer term energy metabolism than it is in acute thermogenic physiological pathways. It also stands to reason that skeletal muscle may be less dependent on non-shivering thermogenic pathways since it can capitalize on shivering thermogenesis in extreme cold.

7.1.4 Leptin

The message specific for leptin was observed in samples of peri-renal adipose tissue, mesenteric adipose tissue, subcutaneous adipose tissue and skeletal muscle. Furthermore we confirmed that significant leptin mRNA was not present in abomasal scraping, despite reports of leptin message in rat and human stomach (Bado et. al., 1998; Sobhani et. al., 2000; Mix et. al., 2000), emphasizing the importance of examining ruminant-specific expression rather than relying on and assuming similarity to findings from monogastric research.

Leptin is one candidate gene that has been studied in several domestic livestock species including to some extent in ruminants. One very recent report indicates that a lower plasma concentration of several hormones including insulin and leptin were associated with higher muscle growth potential in Charolais cattle (Bellmann et al., 2003). An association between a bovine leptin missense mutation (cytosine to thymine transition) and carcass fat content as well as leptin mRNA was recently published, reporting higher carcass fat and increased mRNA in thymine mutant cattle (Buchanan e.t al., 2002). Moreover, Geary et. al. (2003) have suggested that in some cattle serum leptin concentrations are a good positive indicator of carcass composition included calculated yield, marbling score, fat depth and quality grade.

Some breed variation was also noted regarding leptin mRNA abundance in our research project. The leptin mRNA abundance in our mesenteric adipose tissue showed a modest trend (p=0.09) to higher expression in the Charolais steers than in the Angus and Brangus steers. The leptin mRNA in the peri-renal adipose tissue was determined to be significantly lower (p=0.02) in the Angus steers than our other two breeds. Additionally, a breed by feeding level interaction was noted that indicates that leptin mRNA in mesenteric adipose increases with higher feeding levels in the Angus and Brangus steers, as would be expected of the nutrient sensing message. However, the mesenteric adipose leptin mRNA decreased (p=0.03) in our Charolais steers on the 2.2X maintenance diet versus the 1.2X nutritional plane.

Further study regarding relative leptin mRNA abundance in these tissues will be necessary for our understanding of their physiological significance, but our results do accentuate the need to look at tissue-specific expression, in varied commercial breeds prior to making generalizations about the role of leptin in ruminants.

In agreement with the plasma leptin levels and their relationship with carcass characteristics reported by Geary et. al. (2003), we discovered relationships between our leptin mRNA abundance in adipose depots and metabolic parameters. Both our leptin mRNA in subcutaneous adipose (r = 0.41, p=0.02) and mesenteric adipose tissue (r = 0.44, p=0.01) were related to metabolic body weight, and the mesenteric leptin mRNA was further positively correlated with ME of maintenance (r = 0.30, p=0.10). These are interesting observations that strengthen the hypothesis that leptin may serve as an indicator of body energy status in ruminants and may reflect indices of nutrient and metabolic status.

A divergent tissue-specific response was observed in our acute cold experiment where we determined a reduction (p=0.04) in leptin mRNA abundance in subcutaneous adipose cold-treated steers but a trend for increase (p=0.09) in leptin mRNA in perirenal adipose tissue. These differences probably reflect the different adaptation associated with differing tissue-specific exposure to the cold ambient temperature. It's logical to propose that due to the peripheral location of the subcutaneous adipose tissue that this tissue may be exposed to a more dramatic sensation of the acute cold and further that this tissue may respond with increased lipolysis and oxygen consumption which may have influenced the intrinsic expression of the leptin mRNA. In contrast, the exposure of the perirenal adipose tissue to direct sensation of the acute cold ambient temperatures is likely minimal, but the tissue responds to the acute treatment in the context of the animal's visceral systemic response which may be different than that observed in directly cold-exposed tissue.

Finally with regard to observations involving leptin mRNA, an interesting relationship between carcass grade and subcutaneous adipose leptin mRNA was found. In a comparison of mean leptin mRNA abundance in subcutaneous adipose tissue, as grouped according to "A", "AA" or "AAA" carcass grades, there was an inverse relationship (p=0.006). Although this appears to disagree with reports regarding positive plasma leptin concentration associated with improved carcass characteristics (Geary et. al., 2003, Bellmann et. al., 2003), our leptin mRNA abundance determined in this one adipose depot may not relate to a decreased serum leptin concentration. Instead, perhaps the inverse relationship that we observed relates to the specific metabolic status of this adipose tissue. The need to express intrinsic autocrine sources of leptin in subcutaneous adipose tissue may be reduced if the systemic serum leptin concentration is high, as is found in cattle with increased central adiposity, which may reflect the degree of maturity and finishing that translates into an improved carcass grade.

7.1.5 Leptin Receptor

Leptin receptor mRNA was detectable in all of our tissues examined. This relates to the fact that leptin itself may have very systemic effects in terms of its nutrient sensing and its related influence on energy metabolism, this agrees with broad screening strategies in other animal species (Lollman et. al., 1997). In conjunction with its broad dispersal across tissue types we noted considerable variability in tissue-specific leptin receptor mRNA abundance that may ultimately be quite pertinent in our attempts to describe the complexities of its ligand's physiological function.

The only breed effect observed was the trend (p=0.07) showing that Brangus steers show higher leptin receptor mRNA in their cardiac muscle than the other two breeds used for comparison. It will be interesting to further examine whether this increased expression is associated with cardiac function or efficiency, and further whether other *Bos indicus* cattle display this expression profile.

When ADG was statistically compared to the tissue-specific leptin receptor mRNA abundance there were significant correlations identified in both mesenteric adipose tissue (r = 0.43) and subcutaneous adipose tissue (r = 0.30). There was a further positive trend (r = 0.32, p=0.08) between leptin receptor mRNA in subcutaneous adipose and calculated ME of maintenance. These types of associations, although in need of supportive subsequent research, are important in the context of describing the relevance of these genes to the physiology and production characteristics of livestock. Consider the possibility that breeding selection practices selected against higher ME maintenance cattle. It is also possible that we would thus select against higher leptin receptor expression in the subcutaneous adipose tissue. If the associations we observed are validated then leptin receptor mRNA expression in subcutaneous adipose may be in part related to improved average daily gain which producers would likely not wish to select against.

In spite of our observation that leptin receptor mRNA abundance in our skeletal muscle biopsies was affected by an interaction between environment and diet such that, the outside roughage fed steers had the lowest specific mRNA abundance, our acute cold experiment did not significantly alter the leptin receptor mRNA abundance. In our examination of the effect of acute feed restriction we did observe increases in leptin receptor abundance in the duodenum (p=0.06) and the liver (p=0.02). Given the putative role of leptin in nutrient sensing pathways these results were not surprising, and appear especially relevant given the fact that they were observed in tissues related to nutrient absorption and handling. The acute feed restriction may have suppressed the serum leptin concentration, which could have manifested an up-regulation of its ligand specific receptor expression in these visceral tissues.

The physiological regulation of leptin receptor mRNA expression is no doubt complex. The activation of leptin receptor itself acts as an autoregulator of its gene expression since induction of the "Suppressor of Cytokine Signaling (SOCS)" may suppress leptin receptor gene transcription (Bjorbaek et. al., 1998). The regulation of leptin receptor expression will likely prove to be an important factor in the ruminant regarding orchestration of the normal balance between orectic and anorectic signals as well as in the context of its involvement in mediating aspects of systemic and cellular specific energy metabolism.

7.1.6 Neuropeptide Y (NPY)

We did not detect any significant expression of NPY mRNA in any of our screened tissues, but verified the validity and sensitivity of our RT-PCR assay by positive NPY mRNA detection in bovine hypothalamic tissue, the primary site of NPY synthesis. There is also circulating NPY that is released from the sympatho-adrenomedullary nervous system of other animal species (Gray et. al., 1986) but we did not specifically investigate this source in our steers.

7.1.7 Neuropeptide Y Receptor

The mRNA specific for NPY receptor was detected in all our tissues screened except was notably absent in the abomasum. The abomasum represents the only one of our screened tissues that expresses the receptor specific for leptin but not for NPY.

The expression profiles for the NPY receptor in our study, including its broad dispersal across many metabolically active tissues support the premise that it is an important component of the mediation of energy metabolism. The single effect of breed that we found was a trend (p=0.08) toward increased NPY receptor mRNA abundance in subcutaneous adipose tissue of Charolais steers, but not in others. This may again reflect our subjective interpretation that the Charolais were perhaps the most lean of our breeds which might suggest a different metabolic status of their modest quantity of subcutaneous adipose tissue.

We report a positive correlation (r = 0.35, p=0.05) between NPY receptor mRNA in the liver and calculated energy retention values, and further significant decreases in this liver specific NPY receptor mRNA under the influence of both acute cold stress (p=0.03) and acute feed restriction (p=0.01). Therefore an increase in NPY receptor abundance may improve efficiency within these tissue types as they respond with greater sensitivity to the influence of circulating NPY that would be expected to promote energy conservation. Conversely under acute stress it may be a consequence of increased stress hormones and sympathetic activation that serve to reduce the abundance of NPY receptor mRNA in some tissues. Additionally, there was a trend showing reduced NPY receptor mRNA in the skeletal muscle biopsies of our outside long-term acclimated steers versus the indoor-housed steers, and this mRNA was further negatively correlated (p=0.0004) with metabolic body weight. The latter suggests a potential for decrease in muscle NPY receptor mRNA abundance with animal size and maturity.

The NPY receptor mRNA in the cardiac muscle samples was negatively correlated (r = -0.33) with ME of maintenance, whereas this mRNA in subcutaneous adipose is positively correlated (r = 0.38) with this metabolic parameter.

The cumulative interpretation of these results showing varied tissue specific NPY receptor mRNA abundance is difficult at this point, but we are driven to conclude that the expression profile for this gene certainly appears to correlate with several indices of energy metabolism and further are a component of the adaptational strategies implemented by cattle. It is my interpretation that our results support the importance of, and need for, additional evaluation of this candidate gene in terms of its relevance to ruminant energy metabolism.

7.2 Experimental Limitations

Perhaps the primary limitation of this experimental study was our reliance upon mRNA abundance as an indicator of gene expression, it would certainly be beneficial to also characterize the relative protein abundance of these candidate genes. The characterization of both mRNA and protein would further facilitate the analyses of correlations with indices of energy metabolism as well as the physiological adaptation strategies employed by cattle exposed to some typical production scenarios that translate into mild to moderate animal stress. Unfortunately, there are to date no bovine specific antibodies available that could be utilized for the semi-quantification of the bovine specific proteins. It may be possible to try antibodies with epitopes against similar candidate gene proteins from other animal species.

Another limitation of our study was the limited sampling frequency and times, which includes limited accessibility to internal tissues without animal termination. We may have failed to observe many adaptational processes related to our treatments due to our time of sampling, durations and extent of treatment, and lack of sampling of some of our internal tissues. It is possible to develop better sampling methods including use of less invasive laparoscopic tissue acquisition techniques to facilitate further investigation of the expression profiles of these candidate genes. There is always a benefit to performing measures of reference on greater numbers of animals under more stringically controlled though varied conditions to better substantiate correlations, associations and trends. Some parameters such as the unfortunately mild winter we were afforded during our outdoor trial are difficult to control. Though we may be able to utilize artificially cold environments to ensure substantial reduction in ambient temperature we are limited since cold-room space would effectively reduce the number of animals that we could utilize in any long-term acclimation study.

The advent of macro-array and micro-array analyses will facilitate studies such as this one allowing a sensitive means of broad-spectrum analyses of relative abundance of many more candidate genes in many more tissues.

We would certainly benefit from additional characterization of our candidate gene abundance over varied animal age, size, breed, body composition, treatments times, more varied treatments and descriptions and associations with carcass quality parameters. We, as with every other scientist, were most limited by time, manpower and finances but alas such are the realities of research!!!

7.3 General Conclusion

The general conclusion that can be made from this study is that tissue-specific candidate gene expression is varied among individual cattle, varies under the influence of homeostatic and homeorrhetic challenges, differs to some degree from observations made in monogastric species, influences energetic metabolism parameters, and requires and deserves future study so as to better understand ruminant physiology. Finally, utilization of our results and those of subsequent experiments will benefit our livestock production system since knowledge of ruminant specific physiological systems and their adaptation, will direct management and breeding practice so as to optimize production efficiency.

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Appendix One: Tissue Sampling

A.1.1 Biceps femoris Muscle Biopsy

(Developed and implemented by Dr. Artur Cigielski, D.V.M. with assistance of Gordon Murdoch and animal health technologists; Charlaine Gorsak and Brenda Tchir)

Steers were individually prepared for biopsy, by shaving the hair covering the hip to be biopsied. The shaved region (30cmX30cm) was washed with soap and water twice, rinsed with ethanol, and then covered with iodine solution. The steer was then walked by halter to our surgical room and placed in a loose head gate with access to some feed and water throughout the procedure, the tail was loosely tied just so as to prevent its reaching the sterile surgical site. We applied a gentle squeeze to prevent excessive movement only during the incision and biopsy. Local anaesthetic (10-20 ml lidocaine) was injected so as to de-sensitize the region between the ileum and ischium points of the hip.

After waiting 10 minutes to assure effectiveness of local anaesthetic, the area was again treated with iodine solution. A sterile scalpel was used to make a single transdermal incision (approx. 10-14cm), using the hip points as a guide. A sterile surgical probe was utilized to puncture the muscular fascia, and then a finger (surgically scrubbed and gloved) was used to tear the fascia sufficiently to allow access to the underlying *biceps femoris* muscle. Surgical scissors were utilized to quickly and carefully remove between 2.5-5 grams of skeletal muscle. The skeletal muscle biopsy was immediately placed in a piece of pre-labelled (with ear tag and date) aluminum foil quickly weighed, and snap-frozen in liquid nitrogen. The biceps femoris biopsy were transferred to a minus 80 °C freezer and remained frozen until Total RNA extraction.

Soluble internal sutures were utilized to close the skeletal muscle to prevent pocket formation, and an injection of penicillin was introduced to the site of biopsy. The skin was sutured closed and the wound site was sprayed with Borgal. A sterile bandage was placed over the wound and sterile tape was used to seal the bandage to the surrounding skin. The area was cleaned of any trace blood (very limited bleeding occurs) sprayed with K.R.S. to prevent flies and the steer was returned to its pen. The animal was monitored post –op, and all animals recovered completely without a single incidence of infection, abscess or fever.

A.1.2 Post-mortem Tissue collection

Immediately following captive bolt termination and exsanguinations in a licensed abbatoir, the *biceps femoris* was sampled always within three minutes of captive bolt administration. Muscle core samples approximately 100g were cut using a manual 3 cm round stainless steel biopsy tool and immediately packeaged in a labeled 50ml conical tube and snap frozen in liquid nitrogen.

The hide was removed and at the same time 20-50g of subcutaneous adipose was sampled off the hindlimb, placed in a 50ml conical tube and snap frozen in liquid nitrogen.

As the qualified veterinarian inspected the internal organs, perirenal adipose was excised from the outer surface of one of the kidneys, wrapped in labeled aluminum foil and snap frozen in liquid nitrogen.

Simultaneously, approximately 100g samples of cardiac muscle, (CM), liver (L), and mesenteric adipose tissue (MA) were obtained, wrapped in individual pre-labelled aluminum foil and snap frozen in liquid nitrogen.

The duodenum (Duo) was dissected out and a forty to seventy cm portion was removed from the region about five cm below the entrance of the bile duct. This piece of duodenum was then longtitudinally cut, inverted and rinsed lightly in sterile water. The duodenum section was then rinsed sequentially in two tubes of sterile water and then a tube of sterile PBS (pH 7.4). The mucosal surface of the duodenum was the mechanically scraped using a plastic ice scraper. The scrapings were transferred to a pre-labeled fifty ml concial tube and snap frozen in liquid nitrogen.

Next the abomasum was dissected out and inverted such that the content could be rinsed away using a light water wash. The abomaum section was then rinsed sequentially in two tubes of sterile water and then a tube of sterile PBS (pH 7.4). The mucosal lining of the abomasums (ABO) was sampled via several scrapings using a plastic ice scraper. The scrapings were transferred to a pre-labeled fifty ml concial tube and snap frozen in liquid nitrogen.

Finally, a twenty five cm by twenty five cm patch of the ventral rumen was removed and rinsed in a similar fashion as the duodenum and the abomasums. The rumenal papillae, (RUM) were then excised using curved surgical grade scissors, transferred to a fifty ml conical tube and snap frozen in liquid nitrogen.

All samples were transferred to a large cooler and covered with dry ice for transport from the abbatoir to the University. The samples were then immediately transferred to a minus eighty freezer and remained frozen until total RNA extraction.

Appendix Two: Digestion and Energy Metabolism Measurements

A.2. Digestion and energy and nitrogen balance measurements

A.2.1 Body Weight

Animals were weighed (Instaweigh, model 110, Norac Systems International Inc., USA) every second Wednesday morning before feeding throughout the trail. The amount of feed offered to the animals was then adjusted for the following day's feeding based on the result of the body weight measurement.

A.2.2 Urine Collection

During experiments, animals were put into individual metabolic crates (280 cm * 130 cm * 180 cm) equipped with a funnel and screen under the crate for urine collection.

Total urine was collected into plastic buckets containing 200 mL 6 M HCl once each day for three days. After sufficiently mixing and filtrating through Fisher filter paper Q8, urine was proportionally sampled, and then stored at -20 °C. Urine sub samples from the same animal in same period were pooled for assay.

A2.3 Fecal Collection

Fecal samples were collected from pen floor or via rectal palpation at six hours intervals from each calf during three days collection periods. Daily samples from same animal were composited to exclude diurnal variations. After freezed-dry, all fecal and feed samples were ground to pass 1 mm screen in Wiley mill to ensure homogenous sample for analysis. Fecal samples from same animal within period were subsequently pooled in even quantity for DM, GE, acid insoluble ash (AIA) and lignin analysis.

A.2.4 Laboratory Analysis

Duplicate feed and fecal samples were analyzed for DM and ash by standard procedures (AOAC, 1990). Total nitrogen in feed and feces was analyzed by a Leco, FP-428 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI). For measurement of GE in urine, 5 mL urine sample in duplicates was injected into plastic tubing and dried by freeze-dryer. Gross energies of feces and urinary residual and plastic tubing were measured by oxygen bomb calorimetry (AC-300, Leco Instrument Limited, Mississauga, Ontario).

Duplicates of feed and fecal samples were analyzed for AIA by ashing 5 g at 450 °C overnight, boiling in 100 ml 2 N HCl for 5 min, filtering with boiling distilled water, and re-ashing the sample at 450 °C overnight (Van Keulen and

Young, 1977). Lignin content in feed and fecal samples was analyzed in duplicate by the ANKOM method (Komarek, 1993).

A.2.5 Urinary 3-Methyl-Histidine

For 3MH analyses, approximately 200 µL of urine sample or 3MH standard (100 x 10^{-6} M), and 100 µL histidinol (internal standard, 100 x 10^{-6} M) were added into a screw-capped test tube. Samples were deproteinized with 100 µL 3.0 M $HClO_4$ and centrifuged at 3000 RPM for 25 min at 4□C. 200 µL of the supernatant of the deproteinized sample or standard was taken into a 10 mL screw cap vial and then 400 µL H_2O, 100 µL 1.5 M NaOH, and 400 µL 0.2 M $Na_2B_4O_7$ (pH 9.0) were added. While being vortexed, 250 µL fluorescamine (160 mg / 100 mL acetonitrile) was added. Samples were allowed to sit for a few seconds and then 400 µL 2.0 M HCl was added. The vials were mixed, capped, and incubated for 45 min at 90 C in drier oven. Samples were extracted twice with 1.50 mL of diethyl ether. Analysis was performed using a Varian 5500 high performance liquid chromatography system, with a reverse-phase column (Supelcosil 4.6 x 150 mm C18 column) and a Varian 2070 spectrofluorometer detector (excitation 340 nm, emission 450 nm) and a Varian 9090 auto analyzer (Varian Canada Inc.). The mobile phase consisted of a binary gradient changing from a strong polar to a weak polar solvent with a flow rate of 1.0 mL min^{-1}. The polar solvent consisted of 2.5 mM hexadecyltrimethylammonium bromide (HTMA) and 0.1 M sodium acetate buffer (pH 6.50). The non-polar solvent consisted of 10% 2.5 mM HTMA and 90% acetonitrile (pH 6.5). Peaks were recorded and integrated using the Ezchrom^{TM} Chromatography Data System (version 2.12; Shimadzu Scientific Instruments Inc.). The rate of muscle protein degradation (MPD, g/d) and fractional rate of muscle protein breakdown (FBR, %/d) were estimated by urinary 3MH (Gopinath and Kitts, 1984).

A.2.6 Urinary Creatinine

Urinary creatinine concentration was determined by creatinine kits (Sigma Diagnostics, St. Louis, MO 63178) with a spectrophotometer (Spectronic 3000 Array, Milton Roy, USA).

A2.7 Urinary Purine Derivatives (Allantoin)

Allantoin was determined by a modification of the method of Chen et al. (1990). Before actual analysis, urine sample was centrifuged at 3000 RPM for 20 min. The supernatant was diluted 10 times to assay solution. To 0.5 mL of each diluted samples in dulicate and working standards (from 0 to 400 mg allantoin (Sigma A-7878) per litre), 0.6 mL potassium dihydrogen phosphate (0.06M) and 0.3 mL sodium hydroxide (1.8M) was added. Allantoin is hydrolysed under weak alkine conditions to allantoic acid at 85 °C in water bath for 10 min. Then 0.6 mL of HCl (3M) and 0.3 mL of Phenylhydrazine hydrochloride (0.034M) (fresh solution) was added, which allantoic acid is further degraded in weak acidic conditions to urea and glyoxylic acid at 85 °C water bath for 10 min, and the glyoxylic acid is reacted with phenylhydrazine to produce a phenylhydrazone of the acid. After cooling at 10 °C water bath for 5 min, 0.6 mL of HCl (6M) and 0.3 mL potassium ferric cyanide (0.057M) (fresh solution) were added. Reaction with potassium ferricyanide in 20 °C water bath for 5 min yields a chromophore, the color of which is measured at 530 nm by a spectrophotometer (Spectronic 3000 Array, Milton Roy, USA).

A.2.8 Heat Production Determinations by Respiration Calorimetry

Oxygen consumption by the animals was measured (twice in each period) for a 22-h duration by an open-circuit respiration calorimetry system (Mathison et al. 2000), which included, ventilated hoods, a single-channel paramagnetic oxygen analyser (Servomex 540A, Sussex, England), and Beckman infrared CO2 and methane analysers. The steers were previously accustomed to the procedure by a training period of three-four weeks. The system was designed to measure four animals simultaneously. The monitoring period usually started from 1500 h of one day and ended at 1300 of the following day. Before each series of measurements commenced, a complete system calibration was done by injecting a known weight of nitrogen gas into the system. The nitrogen recovery procedure was usually done twice per month or at the beginning and at the end of any series of calorimetry measurements. The nitrogen recovery procedure was done in duplicate on each chamber.

Before the start and at the end of each run, oxygen, CO_2 and methane analyzers were standardized against outside (ambient) air, mixed gases and nitrogen. Air pressure and flow through the system were recorded using a Foxboro electronic pressure/flow transmitter connected to a Strawberry Tree Inc., terminal Panel T41. Air drawn from the animal hoods and the calorimetry room was switched using five Ascolectric switches (Ascolectric Ltd., Brantford ON). Each chamber was ventilated continuously by a 3/4 HP pump (max RPM 1725) (Mathison et al., 2000).

About half-hour to the start of a run, animals were brought from their individual pens into the calorimetry room (room temperature is 15°C) and head placed in the hood. Water, and any left over feed from morning feeding (weighed) was given to the animals in the hood. The amount of left over feed was small. Fresh feed and water were also provided to the animals at 0800 h while the calorimetry system was in operation. Data from the system were collected using the control setup of WORKBENCH PC 2.0 Data Acquisition and Control Software for MS-DOS compatible computers (Strawberry Tree Inc., Sunnyvale, CA). Before the start of each run baseline values for flow and pressure were recorded manually from various digital readouts as well as from the software before and after the ventilation pumps were turned on. During the animal run, values for flow and pressure from the digital display were also recorded at intervals. Additionally, the ventilation rate of the hood (~ 140-160 L min^{-1}) was read manually from a flow meter (Model 10A3555A, Fisher and Porter

Warminster, PA). These provide checks on the electronic recordings collected by the system software software. The time the animals spent lying and standing was recorded using a pressure sensor attached to the floor of the stanchion to correct for possible differences in heat production caused by differences in this activity. No relationship was found between this activity and heat production of the animals and therefore no correction was made to heat production data.

During each 22-h run the ventilation pumps to the four respiration hoods were in continuous operation. A sampling pump was programmed to sample the air outflow from each hood and from room air in sequence for 4 min each, resulting in a recording for each animal once in every 20-min. cycle. The difference in oxygen concentration between room and hood air provided a measure of oxygen usage by the animal. Aliquots of air drawn from chamber were also analysed for CO_2 and CH_4 content using a non-dispersive infrared analyzer (Model 880A, Rosemount Analytical Inc, La Habra, CA). Data generated by the run were processed (corrected to standard temperature and pressure) using an in-house written macro designed to work in a Lotus-123 software environment. Heat production of the animals was calculated from oxygen consumption based on the equation of McLean (1972). Heat Production (fed state) and heat production at zero metabolizable energy intake (fasting HP; derived from regression of HP on MEI for each breed) were used to calculate the partial efficiency of metabolizable energy used for maintenance (K_m) according to the following equation: $K_m = 1 - [(HP - FHP \text{ or intercept})/MEI]$. Metabolizable energy required for maintenance (ME_m) was calculated as FHP (intercept) divided by K_m.

Appendix Three: Study of Gene Expression Using RT-PCR

A.3.1 Sequences for specific gene primers

GENE	**5'to3' Sense primer**	**5'to3' Antisense primer**	**Amplicon size**
UCP-1	agacaggcacatcttcctatcc	cataggactgaagcgattagc	191bp
UCP-2	ctccgtcaagcagttctacacc	atggcattgcgagcgacattg	263bp
UCP-3	tctacgactccgtcaagc	caccatcttcagcatacagtcg	499bp
NPY	atgctaggtaacaagcgactg	cattttctgtgctttctctc	258bp
NPY-receptor	ctgatgagaaccagacagtgg	gtaggtaagtgtaaacggc	313bp
Leptin	tgtatcgattcctgtggctttgg	caacatgtcctgtagtgacc	460bp
Leptin-receptor	gcaactacagatgctctacttt	aaaagttcatccaggccttcttg	379bp

A.3.2 Grinding and total RNA extraction

Tissue samples were collected through surgical biopsy and post-mortem biopsy and snap-frozen in liquid nitrogen to preserve the RNA contained within the tissues. Frozen tissues were ground under liquid nitrogen into fine powders in preparation for total RNA extraction using COORS size 5 ceramic mortar and pestle sets. Tissue samples were utilized as sources of total RNA for the measurment candidate gene messenger RNA expression using RT-PCR, and for the synthesis of two tissue specific bovine libraries (mixed skeletal muscle and mixed adipose). Total RNA from biopsy samples, as well as muscle, fat depots, and tissues from internal organs collected at slaughter was isolated to study the expression of candidate genes. Reverse Transcription- Polymerase Chain Reaction (RT-PCR) was used to analyze gene expression changes in RNA specimens. Our choice of technique was largely determined by transcript abundance and the sensitivity required for detection of the RNA species of interest and we have considerable experience with these methods. Correlations among a variety of energy metabolism indices and gene expression were determined. These measurements were repeated at different stages of production and in animals responding to different diet and environmental conditions.

In order to utilize relative RT-PCR to evaluate the mRNA expression patterns of each specific gene in the various tissues, we first adapted and optimized our protocol for extracting total RNA from all of the tissue types collected. This total RNA was subsequently characterized for integrity using an established and accepted RNA integrity gel method of observing quality of 28S and 18S ribosomal RNA. The total RNA was quantified using spectrophotometric analyses of absorbance at wavelengths of 260nM and 280nM. In all cases non-specific oligo-dT15 reverse transcription was performed to amplify the mRNA in each sample tested using RT-MMLV enzyme (Gibco).The RT-reactions were performed on either a Perkin Elmer 2400 or a Biorad icycler. Each reverse transcription reaction supplied template for a minimum of three specific multiplex PCR reactions using specific primer sets and TAQ polymerase enzyme (Sigma). Sense and antisense primer sets were designed for each of the specific target genes using GenBank as a resource for bovine mRNA sequences. Genejockey and Amplify software were utilized to design and conceptually test each of the primer sets. Our optimized primers sets are able to specifically amplify and thus semi-quantify bovine mRNA representing the following genes; uncoupling protein 1(UCP1), uncoupling protein 2 (UCP2), uncoupling protein 3 (UCP3), leptin (Lep), leptin receptor (leprec), neuropeptide Y (NPY), and neuropeptide Y receptor (NPYrec). Further, we have utilized primers sets for a constitutively expressed gene; glyceraldehydes-3-phosphate dehydrogenase (G3PDH) as our internal control in each of our specific relative reverse transcription polymerase chain reactions (relative RT-PCR). This internal control normalizes for slight variations in reaction efficiency, RNA loading and pipetting. All PCR reactions were performed on either a Perkin Elmer 2400 thermalcycler or Biorad icycler. The products of the PCR reactions (amplicons) were electrophoresed on a 2% agarose /TBE gel along side a molecular weight marker. The gels were stained using ethidium bromide so as to observe the PCR products under UV fluorescence. The specific amplicon bands could thus be relatively quantified generating optical density scores for the amplicon bands using Biorads Gel Doc and Molecular Analyst software. The development of these gene specific primer sets and their associated RT-PCR using protocols will allow better investigation into the physiology of these important genes and their respective products in cattle. In coordination with studies involving these genes in other species, we will better understand the significance in the regulation and variation of these genes in bovine species under varying feed and environmental parameters.

We have found that the fastest and best results were obtained using TRIzol™ reagent (Life Technologies), a monophasic solution of phenol and guanidine isothiocyanate. Therefore TRIzol reagent was utilized to extract the total RNA from the samples after they were ground to a fine powder under liquid nitrogen and dry ice, slight modifications (such as additional and longer centrifugation steps) to manufacturer's recommendation were implemented to improve total RNA yield from difficult tissues such as the adipose.

A.3.2.1 RNA electrophoresis solution

Agarose/Formaldehyde Gel

1. Add 1.5g of agarose into an Erlenmeyer flask
2. Add 130 ml of MQ H_2O
3. Microwave 3 times in 30 second bursts (swirling between) bring to a boil.
4. Add10 ml of 10X MOPS, swirl flask to mix.
5. Allow gel to cool to ~50 to 60°C, then add 8.1 ml of 37% formaldehyde and swirl to mix.

Pour gel into gel unit with combs in place while in fume hood.
Let stand for 1 hour before use.

10 X MOPS

1. 41.85g MOPS (4-morpholinepropanesulfonic acid MW 209.27)
2. 6.80g NaOAc-3 $H_2 0$ (sodium acetate MW 136.08)
3. 20ml 0.5M Na_2EDTA
4. Adjust to pH 7.0 with NaOH
5. Adjust volume to 1 liter
6. Store Protected from light.

RNA loading Buffer

1. 80% formamide
2. 1mM EDTA, pH 8.0
3. 0.1% bromophenol blue
4. 0.1% xylene cyanol

10 X PBS

1. 80g NaCl
2. 2g KCl
3. 26.8g Na_2HPO_4-$7H_2 0$
4. 2.4g KH_2PO_4
5. 800ml $H_2 0$
6. Adjust to pH 7.4 with HCl
7. Adjust volume to 1 liter with $H_2 0$.
8. Sterilize by autoclaving

10 X TBE

1. 108g Tris
2. 55g Boric acid
3. Add 900ml of $H_2 0$
4. 40ml Na_2EDTA, pH 8.0
5. Adjust to 1 liter with $H_2 0$.

2% Agarose Gel

1. Add 2g agarose into an Erlenmeyer flask
2. Add 100 ml of 1 X TBE
3. Microwave 3 times in 30 second bursts (swirling between) bring to a boil.
4. Allow gel to cool to ~50 to 60°C and pour into gel unit

Appendix Four

A.4.1 Feeding and Ort Collection

Orts (refusals) were collected, weighed, sampled and animals were fed daily between 0800 and 0900 h. Representative feed samples were collected whenever a bale of hay was chopped or a new batch of the concentrate mixtures was delivered from the University feed mill. Orts were sampled during the digestibility trials if the amount of feed rejected was more than 5% of the total feed offered. Feed samples and orts were stored at -20°C until laboratory analysis.

A.4.2 Body Weight

Animals were weighed (Instaweigh, model 110, Norac Systems International Inc., USA) every second Wednesday morning before feeding throughout the trail. The amount of feed offered to the animals was then adjusted for the following day's feeding based on the result of the body weight measurement.

A.4.3 Digestibility Trials and Urine Collection

To determine digestibility and urine excretion animals were placed in digestibility crates (2.5 m x 1.1 m x 1.4 m) for 48 hours. The floors of the digestibility crates were made out of metal grates, which allowed the urine to fall through. Below the metal grating was a wire screen that prevented fecal material from contaminating the urine. Below the wire screen was a fiberglass collection tray that funneled urine into a 50l plastic tub, containing 200 ml 6N hydrochloric acid to minimize nitrogen losses, placed beneath it.

Four digestibility crates were used in the indoor environment and 8 crates were used in the outside environment. Animals were tethered to the metabolic crates with the use of a neckband and a chain attached to the front of the crate. Animals were prevented from backing off the crates by placing a chain barrier behind the animal once it was placed on the crate.

Animals were fed as normal while in the metabolic crates, water was provided ad libitum. Water in the outside digestibility crates was prohibited from freezing by the use of an electric water heater in each water container.

A4.3.1 Digestibility Sampling

Fecal grab samples were taken at 0800 h, 1000 h, 1300 h, 1600 h, and 2200 h for each 24 hour period the animals were in the digestibility crates. Fecal samples were pooled and transferred to a freezer at -20°C until further analysis.

A4.3.2 Urine Sampling

Urine excretion was weighed and sampled at 0800 h for each 24 hour period the animals were in the digestibility crates. Representative urine samples (5% w/w) were taken from each day's total excretion, filtered, pooled and transferred to a freezer at -20°C until further analysis.

A.4.3.3 Laboratory Analysis

Feed and fecal samples were dried to a constant weight at 100°C and ground through a 1 mm screen with Thomas Wiley laboratory mill (model 4, Philadelphia, USA). They were then analyzed for DM, ash, nitrogen content, fiber (neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose, hemicellulose and lignin), acid insoluble ash (AIA) and gross energy (GE).

Urine samples were analyzed for allantion concentration and gross energy. Before GE content of urine was determined the samples were dried by freeze drying (Virtis company 50-SRC freeze dryer, Gardiner, NY, USA). The gross energy content of the urine samples was determined by adiabatic bomb calorimetry (Leco automatic calorimeter AC300, St. Joseph, MI, USA).

A.4.3.4 Fiber Analysis

NDF, ADF, hemicellulose, cellulose, ash, and lignin concentrations in feed, feces were determined by a modified filtering procedure described by Van Soest and Robertson (1980). The filter bag technique developed by the ANKOM company was used for the analysis of NDF, ADF, hemicellulose, cellulose and ash (Ankom Company, 1993).

A.4.3.5 Nitrogen Analysis

Nitrogen was determined by an automated nitrogen analyzer (LECO).

A4.3.6 Energy Analysis

Gross energy of feed, feces and urine samples was determined by adiabatic bomb calorimetry (Leco automatic calorimeter AC 300, St. Joseph, MI, USA).

A.4.3.7 Allantion Analysis

Allantoin was determined by a modification of the procedure described by Chen et al. (1990)., as described for experiment one

A.4.4 Energy expenditure from Oxygen Utilization, Carbon Dioxide and Methane Production

After the animals completed the digestibility and urine collection trials they were returned to their respective pens. Respiration measurements were taken 3 days after the completion of the digestibility trials, using a five channel indirect calorimeter, allowing measurements to be done on 4 animals at a time. During calorimetry the animals were housed in metabolic chamber maintained at a thermoneutral temperature (9.6°C ± 3.4°C). For oxygen, carbon dioxide (CO_2) and methane (CH_4) measurements the animals were held in calorimetry stanchions (2 m x 0.9 m) a total of 23 hours and data was collected for the last 22 hours. Air was continuously withdrawn from each of the four hoods, and gas flow, temperature, relative humidity, pressure, and O_2, CO_2 and CH_4 concentration were all monitored according to procedures described earlier.